Healing of grafts of Bio-Oss® and Moa-Bone®

in a sheep maxillary sinus model

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For Sarah, Max, Poppy and Zac
Abstract

Maxillary sinus grafting to increase the bony support available prior to dental endosseous implants is a common surgical procedure. Autogenous bone has historically been considered the gold standard for maxillary sinus augmentation. Xenograft materials such as Bio-Oss® (BO) are commonly used alternatives to autogenous bone. Moa-Bone® (MB), a xenograft material similar to BO, may provide an alternative to BO. Reasons for differences in the healing of grafting materials in augmented maxillary sinus sites are poorly understood.

Aim: To characterize the early healing of Bio-Oss® and Moa-Bone® in a sheep maxillary sinus model, using cellular markers for osteoblasts, osteoclasts and proliferation.

Methodology: Two animals were selected to represent healing time intervals of four, six, eight and 12 weeks after sinus augmentation with either BO or MB. Animals were sacrificed and specimens from the augmented site resected en bloc. Specimens were divided in two and either embedded in resin, ground and stained with MacNeal’s tetrachrome/toluidine blue, or decalcified and embedded in paraffin, sectioned in a step-serial manner and stained with H&E. Paraffin-embedded tissues were examined using immunohistochemical staining for RUNX2 and PCNA or TRAP histochemical staining for osteoclasts, conducted.

Results: The pattern of healing of sites grafted with the test materials differed considerably. BO site were characterized by early formation of bone and minimal resorption of BO. The earliest bone formed around BO particles closest to pre-existing bone. No resorption of the BO particles was observed. Early healing in MB grafted sites was characterized by large numbers of osteoclasts and obvious resorption of MB material. Large numbers of differentiating osteoblasts were observed in the connective tissue surround MB particles from six weeks of healing onwards. Both materials were surrounded by bone after 12 weeks of healing.

Conclusion: Both BO and MB are associated with new bone formation after 12 weeks of healing in the sheep maxillary sinus. The healing of MB grafted sites is characterized by obvious resorption of graft particles and large numbers of cells with osteogenic potential. Future investigations will determine whether the presence of these cells will positively impact on dental implant integration.
Acknowledgements

Associate Professor Warwick Duncan, my principal supervisor, has invested considerable time and energy into this project. Without his knowledge of working with the sheep animal model this project would not have been possible.

The development of the laboratory techniques would have been impossible without the advice and assistance of Dr Dawn Coates. She has encouraged me at every opportunity, in the laboratory and during preparation of the manuscript, for which I am deeply indebted. I am also very grateful for the invaluable support of Professor Gregory Seymour.

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## Table of contents

Abstract .................................................................................................................................................. ii
Acknowledgements ............................................................................................................................... iii
Table of contents .................................................................................................................................... iv
List of figures ........................................................................................................................................... xi
List of tables .......................................................................................................................................... xiv
List of Abbreviations and symbols ..................................................................................................... xv

Chapter 1 Introduction and literature review ....................................................................................... 1

1.1 The maxillary sinus ........................................................................................................................... 3

1.1.1. Human sinus anatomy ............................................................................................................... 3

1.1.2. Animal sinus anatomy ............................................................................................................... 3

1.1.3. Alveolar resorption ................................................................................................................... 3

1.1.4. Sinus pneumatisation ............................................................................................................... 4

1.1.5. Summary ....................................................................................................................................... 5

1.2. Sinus grafting and dental implants ................................................................................................ 6

1.2.1. Caudwell-Luc operation .......................................................................................................... 6

1.2.2. Modified lateral approaches .................................................................................................. 7

1.2.3. Trans-crestal approach .......................................................................................................... 7

1.2.4. Alternatives to sinus grafting .................................................................................................. 7

1.2.5. Piezo – electric devices .......................................................................................................... 9

1.2.6. Summary ....................................................................................................................................... 10

1.3. Implants in the grafted sinus ........................................................................................................ 10

1.3.1. Survival of implants in augmented sinus sites ........................................................................... 10

1.3.2. Delayed versus immediate placement ..................................................................................... 10

1.3.3. Influence of implants on healing of grafted sites ..................................................................... 11

1.3.4. Summary ....................................................................................................................................... 11

1.4. Animal Models ................................................................................................................................ 12
1.4.1. Large animals ................................................................. 13
1.4.2. Small animals .................................................................. 15
1.4.3. Primates ........................................................................... 16
1.4.4. Validity of animal models .................................................. 16
1.4.5. Summary .......................................................................... 16
1.5. Bone Biology ........................................................................... 18
  1.5.1. Normal bone ................................................................. 18
  1.5.2. Healing bone ................................................................... 20
  1.5.3. Graft consolidation ........................................................... 21
  1.5.4. Osteogenesis .................................................................. 22
  1.5.5. Osteoinduction ................................................................. 22
  1.5.6. Osteoconduction ............................................................... 23
  1.5.7. Angiogenesis .................................................................. 23
  1.5.8. Gradient of consolidation .................................................. 23
  1.5.9. Resorption ...................................................................... 24
1.6. Sinus grafting materials ........................................................... 25
  1.6.1. Autografts ..................................................................... 25
  1.6.2. Allografts ...................................................................... 25
  1.6.3. Alloplastic (synthetic) materials ........................................ 26
  1.6.4. Xenografts ..................................................................... 27
  1.6.5. Summary ........................................................................ 28
1.7. DBB products .......................................................................... 28
  1.7.1. Physico-chemical differences between materials ................. 29
  1.7.2. Reduction in carbonate groups ......................................... 29
  1.7.3. Sintering ........................................................................ 29
  1.7.4. Porosity .......................................................................... 30
2.3.2. Moa-Bone® ................................................................. 54
2.3.3. Bio-Gide® ................................................................. 55
2.3.4. Piezotome™ .............................................................. 56
2.3.5. Hand instruments ....................................................... 56
2.4. Surgical protocols .......................................................... 56
2.4.1. Anaesthetic management .............................................. 56
2.4.2. Surgical pain control ................................................... 56
2.4.3. Surgical procedure ..................................................... 57
2.4.4. Post-operative pain control .......................................... 60
2.4.5. Euthanasia ................................................................. 60
2.4.6. Perfusion protocol ...................................................... 61
2.4.7. Harvesting ................................................................. 62
2.5. Resin-embedded tissue .................................................... 64
2.5.1. Resin-embedding protocol ........................................... 64
2.5.2. Sectioning of resin-embedded tissue .............................. 65
2.5.3. Staining ................................................................. 67
2.6. Paraffin-embedded tissue .................................................. 68
2.6.1. Decalcification .......................................................... 68
2.6.2. Sectioning and slide preparation ................................... 70
2.6.3. H and E ................................................................. 70
2.6.4. TRAP (Tartrate Resistant Acid Phosphatase staining) ....... 70
2.6.5. Counterstaining and Coverslip ..................................... 70
2.7. Immunohistochemistry .................................................... 72
2.7.1. Pre / post - treatment(s) ............................................... 72
2.7.2. RUNX 2 immunohistochemistry ................................... 74
2.7.3. PCNA immunohistochemistry ..................................... 74
2.7.4. Control tissues ................................................. 74
2.7.5. Secondary antibodies and development of HRP ......................... 75
2.8. Imaging of histological sections ........................................ 76
  2.8.1. Flat bed scanning .............................................. 76
  2.8.2. Microscopy ........................................................ 76
Chapter 3 Results .................................................................. 79
  3.1. Handling properties ..................................................... 79
  3.2. Post-operative recovery ............................................... 79
  3.3. Site harvesting .......................................................... 80
  3.4. SEM examination ....................................................... 81
  3.5. Radiographic examination of specimens .......................... 84
  3.6. Examination of resin-embedded sections ........................ 86
     3.6.1. Description of histology for resin-embedded sections .... 89
  3.7. Examination of decalcified paraffin-embedded sections .......... 95
     3.7.1. Control Tissues ................................................... 96
     3.7.2. Descriptions of healing around graft particles near to pre-existing bone ........ 99
     3.7.3. Descriptions of healing around graft particles distant from pre-existing bone ... 112
  3.8. Summary of analysis for decalcified paraffin-embedded tissue ...... 126
     3.8.1. Bio-Oss® .......................................................... 126
     3.8.2. Moa-Bone® ....................................................... 126
Chapter 4 Discussion ............................................................... 128
  4.1. Introduction ................................................................ 128
  4.2. Differences in healing of maxillary sinus grafts using BO or MB .... 128
  4.3. The experimental model ............................................... 131
  4.4. Experimental findings as compared to other models ................ 132
  4.4. Bone modeling and remodeling ..................................... 137
4.5. Bone formation ........................................................................................................... 137
4.6. Resorption .................................................................................................................. 138
4.7. Gradient of healing ..................................................................................................... 140
4.8. Relationship between resorption and bone formation ............................................. 141
4.9. Clinical significance .................................................................................................. 142
4.10. Confounding factors and other issues with the investigation ................................. 144
4.11. Conclusions and recommendations for further research ....................................... 147
  4.11.1. Conclusions ........................................................................................................ 147
  4.11.2. Characterization of healing sinus grafts ........................................................... 147
  4.11.3. Investigation of the materials in different formats ............................................ 148
  4.11.4. Investigations concerning dental implants ......................................................... 149
  4.11.5. Investigations concerning the osteoinductive potential of MB ....................... 149
References ......................................................................................................................... 150
Appendices .......................................................................................................................... 170
Appendix I .......................................................................................................................... 170
  1. Chemical Reagents used .......................................................................................... 170
  2. Kits ............................................................................................................................. 171
  3. Equipment .................................................................................................................. 171
  4. Materials and medications used in sheep surgery ..................................................... 172
  5. 0.1M Sodium Citrate Buffer Stock Solution ............................................................ 174
  6. Scotts Water ............................................................................................................... 175
  7. Ethylenedinitrilotetraacetic acid (EDTA) solution .................................................... 175
  8. Saturated Solution of Ammonium Oxalate: .............................................................. 176
  9. Serum Heat Treatment .............................................................................................. 176
Appendix II .......................................................................................................................... 177
  1. Resin for embedding ................................................................................................. 177
2. Resin embedding protocol ................................................................. 178
3. Staining with MacNeal's Tetrachrome / Toluidine Blue solution .......... 179

Appendix III ............................................................................................ 180
1. Specimen Decalcification Protocol ...................................................... 180
2. Ammonium Oxalate Test for Decalcification ...................................... 180
3. Specimen Orientation ........................................................................ 180
4. Paraffin embedding process for decalcified specimens ..................... 181
5. Immunohistochemical Agents Used ................................................... 183
6. Haematoxylin and eosin routine staining .......................................... 184
7. Immunohistochemistry for TRAP ...................................................... 185
8. Immunohistochemistry for RUNX2 ................................................... 188
9. Immunohistochemistry for PCNA ...................................................... 194
List of figures

Figure 1 Regulation of osteoblast differentiation by RUNX2, Sp7 and canonical Wnt signaling 19
Figure 2 Overview of events surrounding cellular differentiation during graft consolidation ..... 22
Figure 3 Relative expression of cellular markers during osteoblast differentiation .................. 37
Figure 4 Bio-Oss®, in supplied sterile glass vial ................................................................. 54
Figure 5 Bio-Gide® membrane ............................................................................................ 55
Figure 6 Diagram of a dry sheep skull, identifying location of surgical site ...................... 57
Figure 7 Surgical bony window access to the maxillary sinus ............................................. 58
Figure 8 The test materials immediately prior to surgical placement ................................. 59
Figure 9 Application and placement of grafting material ..................................................... 59
Figure 10 Placement of Bio-Gide® membrane (1st layer) ..................................................... 60
Figure 11 Radiograph of specimen to identify location of graft material prior to sectioning ...... 63
Figure 12 Diagram of specimen sectioning .......................................................................... 64
Figure 13 Sequence of specimen processing ........................................................................ 66
Figure 14 Decalcified paraffin-embedded section showing the two regions of interest .......... 77
Figure 15 SEM images of graft materials at low magnification ........................................... 81
Figure 16 SEM images of graft materials at medium magnification ...................................... 82
Figure 17 SEM images of graft materials at high magnification ........................................... 83
Figure 18 Radiographic comparison of harvested specimens .............................................. 84
Figure 19 Resin-embedded tissue, near to pre-existing bone, at four weeks ....................... 91
Figure 20 Resin-embedded tissue, distant from pre-existing bone, at four weeks ............... 91
Figure 21 Resin-embedded tissue, near to pre-existing bone, at six weeks ......................... 92
Figure 22 Resin-embedded tissue, distant from pre-existing bone, at six weeks ................ 92
Figure 23 Resin-embedded tissue, near to pre-existing bone, at eight weeks ....................... 93
Figure 24 Resin-embedded tissue, distant from pre-existing bone, at eight weeks ............. 93
Figure 25 Resin-embedded tissue, near to pre-existing bone, at 12 weeks ....................... 94
Figure 26 Resin-embedded tissue, distant from pre-existing bone, at 12 weeks ............... 94
Figure 27 Comparison of resin-embedded and decalcified-paraffin-embedded sections ...... 95
Figure 28 Control Sections for RUNX2 Staining................................................................. 96
Figure 29 Control Sections for PCNA Staining ................................................................... 96
Figure 30 H&E staining at 4 weeks, near to pre-existing bone ........................................... 100
Figure 56 Immunohistochemical labeling with RUNX2 at 12 week distant from pre-existing bone
.................................................................................................................................................................. 124

Figure 57 Immunohistochemical labeling with PCNA at 12 weeks, distant from pre-existing bone
.................................................................................................................................................................. 125
List of tables

Table 1 Relative similarity of animal bone structure to human bone structure.......................... 12
Table 2 Summary of commonly used techniques for analysis of healing osseous grafts .......... 35
Table 3 Studies included in this review of histological reports of membrane protected maxillary sinus sites grafted with Bio-Oss®, without simultaneous implant placement in humans .......... 43
Table 4 Summary of histological findings from this review of reports of membrane protected maxillary sinus sites grafted with Bio-Oss®, without simultaneous implant placement in humans .................................................................................................................. 44
Table 5 Slide staining scoring system ....................................................................................... 78
Table 6 Summary of histological observations for resin-embedded sections where Bio-Oss® was located in the two regions of interest .................................................................................................................. 87
Table 7 Summary of histological observations for resin-embedded sections where Moa-Bone® was located in the two regions of interest ............................................................................................................. 88
Table 8 Summary of histological observations for decalcified paraffin-embedded specimens where Bio-Oss® graft material was located near to pre-existing bone ............................................................ 97
Table 9 Summary of histological observations for decalcified paraffin-embedded specimens where Moa-Bone® material was located near to pre-existing bone .......................................................... 98
Table 10 Summary of histological observations for decalcified paraffin-embedded specimens where Bio-Oss® was located distant from pre-existing bone ......................................................... 110
Table 11 Summary of histological observations for decalcified paraffin-embedded specimens where Moa-Bone® material was located distant from pre-existing bone .............................................. 111
Table 12 Summary of specimen processing ............................................................................... 173
Table 13 Paraffin-embedded tissue, slide staining plan ............................................................... 182
### List of Abbreviations and symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>ARF</td>
<td>Activation-Resorption-Formation (cycle)</td>
</tr>
<tr>
<td>AEC</td>
<td>animal ethics committee</td>
</tr>
<tr>
<td>APES</td>
<td>3-aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>AWO</td>
<td>animal welfare officer</td>
</tr>
<tr>
<td>BG</td>
<td>Bio-Gide®</td>
</tr>
<tr>
<td>BO</td>
<td>Bio-Oss®</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSP</td>
<td>bone sialoprotein</td>
</tr>
<tr>
<td>Cbfa1</td>
<td>core binding factor alpha</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>CT</td>
<td>connective tissue</td>
</tr>
<tr>
<td>DAB</td>
<td>3, 3’ diaminobenzidine</td>
</tr>
<tr>
<td>dpi</td>
<td>dots per inch</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii (latin = and others)</td>
</tr>
<tr>
<td>GBR</td>
<td>guided bone regeneration</td>
</tr>
<tr>
<td>GmbH</td>
<td>Gesellschaft mit beschränkter Haftung (German =Limited Company)</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HRP</td>
<td>streptococcal horse radish peroxidase</td>
</tr>
<tr>
<td>i.m.</td>
<td>intra-muscular</td>
</tr>
<tr>
<td>i.v.</td>
<td>intra-venous</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
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<tr>
<td>IgG</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>Ltd</td>
<td>limited (company)</td>
</tr>
<tr>
<td>M</td>
<td>molar (concentration)</td>
</tr>
<tr>
<td>MB</td>
<td>Moa-Bone®</td>
</tr>
<tr>
<td>mcg</td>
<td>microgram</td>
</tr>
<tr>
<td>mm</td>
<td>millimetre</td>
</tr>
<tr>
<td>MMA</td>
<td>methyl methacrylate</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NBF</td>
<td>neutral buffered formalin</td>
</tr>
<tr>
<td>NFMP</td>
<td>non-fat milk powder</td>
</tr>
<tr>
<td>NZ</td>
<td>New Zealand</td>
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<tr>
<td>OC</td>
<td>osteocalcin</td>
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<tr>
<td>ON</td>
<td>osteonectin</td>
</tr>
<tr>
<td>OP</td>
<td>osteopontin</td>
</tr>
<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PDL</td>
<td>periodontal ligament</td>
</tr>
<tr>
<td>pH</td>
<td>negative logarithm of hydrogen ion concentration</td>
</tr>
<tr>
<td>Pty</td>
<td>proprietary limited company</td>
</tr>
<tr>
<td>qRT²PCR</td>
<td>quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RANK</td>
<td>receptor activator of nuclear factor - κβ</td>
</tr>
<tr>
<td>RANKL</td>
<td>ligand of receptor activator of nuclear factor - κβ</td>
</tr>
<tr>
<td>rhBMP</td>
<td>human recombinant bone morphogenic protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RUNX2</td>
<td>runt-related transcription factor 2</td>
</tr>
<tr>
<td>s.i.d.</td>
<td><em>semel in die</em> (latin = once per day)</td>
</tr>
<tr>
<td>SM</td>
<td>Schneiderian membrane</td>
</tr>
<tr>
<td>TCP</td>
<td>tri-calcium phosphate</td>
</tr>
<tr>
<td>TRAP</td>
<td>tartrate resistant acid phosphatase</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>ZNL</td>
<td>Zweigniederlassung (German = affiliate)</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction and literature review

The restoration of alveolar defects in edentulous areas prior to prosthetic rehabilitation is a common clinical problem. Historically it was common to replace missing teeth on a tooth supported partial denture. The advent of endosseous implants has expanded the treatment choices for replacing missing teeth and patients are increasingly electing an implant-based solution in edentulous sites. However lack of sufficient bone, due to the anatomy of the posterior maxilla, and bony remodeling following extraction of teeth often complicates the use of dental implants. This is due to either bony atrophy [1-3] or pneumatisation of the maxillary sinus space. Furthermore, in the region of the maxillary sinus the quality of the residual maxillary bone is often poor and the resultant edentulous space of insufficient height, with respect to implant placement [4]. Bone lost following extraction is a barrier to planning aesthetic and functional implants [5].

Maxillary sinus grafting has been significantly developed, since the first reports in the 1980s [6, 7]. Originally a pre-prosthetic procedure [8], sinus grafting is now most commonly performed to increase the bone available to receive a dental implant. Augmentation of the maxillary sinus may be required for the successful placement of maxillary dental implants [9], and a large number of materials and techniques have been described with a view to accomplishing this goal. Recent literature reviews have examined the success of biomaterials and implants currently used in sinus augmentation, and are unanimous in their recommendation of increased research in this field [9-11]. Despite many clinicians holding to the view that autogenous bone is the preferred augmentation material in the maxillary sinus, there are ongoing issues with the harvesting of sufficient material for adequate augmentation. Additionally, the increased morbidity associated with a second surgical site has led to interest in predictable alternatives to autogenous bone. The bovine derived xenograft product Bio Oss® (BO) is probably the most commonly utilized, and the most studied [10]. BO has recently been referred to as the ‘gold standard’ amongst bone graft substitute materials [12].

Despite the popularity of BO, there remain many aspects of its biological behavior which are poorly understood. Not least among these is the question of whether it is resorbed during graft
consolidation and whether sites grafted with BO are subject to normal bone modeling and re-modeling. It is within this context that animal models have been developed which aim to gather information which cannot be obtained from biopsy of human tissues.

New materials, similar in origin \cite{13} and chemistry \cite{14} to BO are regularly reported in the dental literature. Moa Bone® (MB) is a bovine derived material currently used in ophthalmic prosthetic reconstructions. MB has a very similar bio-chemical profile to BO \cite{15}. It is approved and certified for use in Europe and the USA, and is subject to the same strict regulation as any medical product.

The objective of this investigation is to compare the healing of maxillary sinus sites grafted with either BO or MB in an ovine model.
1.1 The maxillary sinus

Historically, manipulation of the maxillary sinus space has been avoided or restricted to the field of otolaryngology. Grafting of the sinus region was limited to post-traumatic interventions \[^{16}\]. Interest in surgical manipulation of the region, for dental purposes, began with pioneering surgery in the 1960’s, before the advent of modern implant techniques and the widespread implementation of osseointegrated, titanium dental implants \[^{17}\].

1.1.1. Human sinus anatomy

The maxillary sinus is a pyramidal space situated in the body of the maxilla \[^{18}\]. The sinus floor is formed in part by the maxillary alveolar process and the hard palate. The floor is made of dense lamellar bone which can be topographically smooth, or undulating as it interacts with the root tips of the posterior maxillary dentition. The sinus floor may dehisce, following pneumatisation in the sinuses of older individuals, with only the sinus lining membrane covering the root apices \[^{19}\], or paper thin antral floor, in the case of an edentulous ridge \[^{20}\].

The sinus lining membrane, also known as the Schneiderian membrane (SM), is a mucous membrane in direct interaction with environmental air, via the ostium and the nose. The SM consists of thin, ciliated, columnar, respiratory epithelium in very close approximation to the connective tissues and blood vessels of the underlying periosteum. The SM is between 0.13mm and 0.5mm in thickness in humans \[^{21, 22}\].

The arterial supply to the maxillary sinus is via the branches of the maxillary artery. Microangiographic analysis has determined that the vasculature of the region exhibits a high degree of microanastomosis \[^{23}\]. Significantly for the re-vascularisation and healing of grafts in the region, there is a decrease in the blood supply, in terms of number and quality of vessels, following removal of adjacent teeth and in advancing age \[^{24}\].

1.1.2. Animal sinus anatomy

The suitability of the sinus anatomy in the available animal models is discussed in section 4.

1.1.3. Alveolar resorption

Changes in bone quality and quantity are derived from alterations to physiologically normal modeling and remodeling. Modeling describes the process of adapting to increased or decreased strain. Remodeling describes the process of natural turnover of healthy bone. The resorption of
the residual alveolus is a common observation following dental extractions, although the extent of the resorption is highly variable, depending on many factors including site and time since extraction \[^{25, 26}\]. The process is considered to be a ‘disuse atrophy’, whereby the loss of the tooth reduces strain on the surrounding bone. This results in adverse modeling of the region \[^{27}\], with respect to bone preservation. The physiological loss of bone is determined by Wolff’s Law \[^{28}\], whereby bone volume and architecture will change according to the loads placed upon it. The precise reasons for variability in extent and rate of resorption are poorly understood. The means of transmission of strain to the alveolus in the dentate individual is via the periodontal ligament, which is severed during dental extraction. The integration of dental implants provides some strain stimulus to the residual alveolus, however the implications for bone modeling are not well known, although increased density of peri-implant bone has been described \[^{29, 30}\]. Peri-implant bone appears to be more cortical in nature regardless of the type of bone that the implant is inserted in to, indicating the importance of strain distribution in determining the nature of bony remodeling.

Techniques designed to reduce post extraction modeling, otherwise known as ‘ridge preservation’, include atraumatic extraction and the use of filler materials. For recent review of ridge preservation techniques see Darby et al. (2008) \[^{31}\].

**1.1.4. Sinus pneumatisation**

The same principles of that underlie the process of bone modeling and remodeling can also result in the phenomenon known as pneumatisation. The sinus space increases in size, due to the forces and strains upon the sinus floor. This is particularly so in the absence of teeth, when forces resulting from mastication are no longer transmitted to the deep tissues of the alveolus \[^{16}\]. The extent of pneumatisation of the sinus region is related to the duration of edentulousness \[^{32}\] and may adversely affect the quantity of basal bone available for implant stabilization \[^{33}\]. The pneumatised sinus may also expand to fill space previously occupied by teeth. It was this clinical scenario which led to the first sinus grafting surgeries, rather than as a pre-implant technique. The goal of this technique was to reduce the size of the posterior maxilla prior to prosthetic restoration \[^{8}\].
1.1.5. Summary

The twin phenomena of alveolar atrophy and sinus pneumatisation often result in the region having insufficient of bony tissue for simple implant placement; requiring augmentation of the sinus space.
1.2. Sinus grafting and dental implants

A number of different approaches to augmenting the sinus region have been described. Initially these procedures were designed to correct unfavourable alveolar ridge morphology prior to restorations with removable prostheses. The procedure has been adapted to the evolving technologies of integrated dental endosseous root-form implants.

1.2.1. Caudwell-Luc operation

The development of maxillary sinus surgery as a pre-prosthetic procedure was first advocated by Boyne in the 1960’s, although this was primarily to allow for provision of traditional removable partial dentures [6]. The removal of the expanded, or pneumatised, tuberosity was complicated by a lack of underlying bone. For this reason, the bony lateral wall of the antrum was exposed and a ‘window’ created to expose the sinus lining. The SM was elevated from the internal antrum wall, or floor, of the sinus and the resultant space packed with harvested autogenous bone. The site was further modified, as necessary at a later time, to achieve a ridge morphology suited to removable denture placement. Known as a Caudwell-Luc approach this is identical to techniques used in the first published literature concerning the placement of dental endosseous implants in the augmented maxillary sinus [6].

The use of dental implants, following the discovery and development of osseointegration [17], increased attention to bony augmentation of the posterior maxilla. Early studies had indicated the positive effect of increased implant length on implant survival [34, 35], although the actual height of residual bone required to successfully support an implant has remained contentious [36]. A number of techniques have been developed to achieve the goal of adequate quantities of hard tissue for implant placement, using a variety of surgical approaches [37]. The first reported graft material was autogenous bone, whether cancellous, cortical or a mixture of both. The graft material may also have been block or particulate form [38]. This lead to the conclusion that autogenous bone was deemed ‘appropriate for sinus grafting’ [36], based on the analysis of nearly 3000 implants placed in just over 1000 grafted sinuses with a 5 year survival rate of 92%. Further reviews confirmed the utility of autogenous materials, although ‘combination’ grafts were also associated with high implant survival rates [2, 39]. The combination graft, consisting of autogenous bone in conjunction with another bio-material, decreased the amount of autogenous bone required,
potentially decreasing surgical morbidity, complexity and cost. These alternative bio-materials will be discussed in detail in section 3.8.

1.2.2. Modified lateral approaches
The Caudwell-Luc technique has been modified for dental implant purposes, using anatomically inferior access point, adjacent to the level of the residual alveolar ridge, and thus the site to be augmented [37]. However, this lower position has been assessed as more vulnerable to post-operative infection due to natural drainage over and into the augmented site [16]. Further variations to the ‘lateral’ approach include removal, repositioning or in-facturing of the bony window created in the antrum wall [40] and partial or full elevation of the sinus lining. Each of these techniques has advocates, depending on the skills and experience of the surgeon involved [36]. The use of membranes to cover the graft will be discussed in section 1.8.

1.2.3. Trans-crestal approach
Summers [41, 42] described modifications of the approach which utilized osteotomes to condense and displace the local bone overlying the sinus floor. This was suggested to improve the bone quality lateral to the site and elevate autogenous bone vertically into the uplifted sinus. The technique is reportedly very successful [43]. Implants had similar survival rates over similar time periods as for un-augmented sites. However, the analysis did indicate that the technique was dependant on sufficient ridge height to gain primary stability, approximately 5mm. This would indicate that where ridges are four mm or less, that lateral augmentation should be considered as a more predictable alternative to the trans-crestal approach [16].

1.2.4. Alternatives to sinus grafting

1.2.4.1. Short implants
Recent publications have investigated the possibility of the use of short implants, less than 10 mm long, in order to avoid the need for sinus augmentation [44–46]. Roughened surface implants are associated with improved survival rates in the sinus region [3]. New techniques and novel implant designs [47] may reduce the need for augmentation. However the success of implants placed into augmented sinus sites indicates that the technique will be used in conventional implant dentistry for the foreseeable future [2, 9].
1.2.4.2. Sinus lift without graft material

Recent human studies [48-50, 51], have confirmed previous observations in humans [52] and sheep [53] that it may be possible to induce new bone beneath the elevated SM in the absence of filler material. In these studies the apex of the implant was placed so that it ‘tents up’ the SM, allowing new bone to fill the resultant space created between the SM, the implant and the sinus floor. In contrast, doubts have been raised whether the amount of bone gained is clinically significant [54].

The retrospective study by Schmidlin et al. (2008) [51], reported 100% survival rates, after a mean follow-up period of 17.6 months, for implants placed in to non-augmented sinus sites. The average initial residual bone height was radiographically assessed at approximately 5mm. The implants were functionally loaded at six months and follow up radiographs indicated considerable bone fill of nearly 90% around the apical portions of the implants.

Interestingly, in the study by Palma et al (2006) [50], the authors noted significant differences between the healing of the blood clot around implants with different surfaces. Machined surface implants were associated with less bone than implants with a roughened surface. The study by Boyne et al. (1993) [52] also noted that changes in the morphology of the apical portion of the implant affected the amount of new bone created using this technique. Round-ended implants were associated with significantly more bone than those with sharp margins, open apices and deep threads [52].

These differences in healing indicate difficulties in comparing much of the implant literature, where different implant systems report different results for similar procedures. In the absence of an implant or slowly resorbed graft material to maintain the space created by elevating the SM, it appears that the clot will quickly resolve and the shape of the membrane returns to its pre-surgical state [55].

There is conflicting evidence whether the cells of the SM have the ability to induce osteogenesis [54]. Osteoprogenitor cells were located in human SM tissue in vitro, as well as new bone induced when these cells were transplanted in association with a ceramic hydroxyapatite / tri-calcium phosphate graft particle [56]. Whether these potentially bone forming cells are elevated with the SM, or remain in contact with the bony sinus floor was not investigated, but the authors acknowledged the possibility that these cells may form part of a vital periosteum, and thus would be expected to be found in these locations. There was no sign of osteogenic activity from the
elevated SM at any point during the 30 days of healing reported in the recent study by Scala et al. (2010) using a capuchin monkey model [54].

1.2.5. Piezo – electric devices
Traditionally, following mucoperiosteal flap elevation, the bony antral wall of the sinus has been removed using a surgical bur or trephine, regardless of whether the bone is entirely ablated, in-fraughted or removed and replaced after grafting is complete. It has been suggested that the use of a bur or trephine will generate heat, even with water cooling, which may impair healing and may also be associated with unacceptably high rates of accidental trauma to the tissue close to the surgical site [57-59]. Recently there has been increased use of piezoelectric devices for cutting of bone, such that the technique has been described as ‘cutting edge’ technology for cranio-facial bone surgery [60]. These devices are claimed to be less destructive to the adjacent tissues and be more easily controlled in delicate situations [61, 62]. The design of the piezo tips also limits damage to surrounding soft tissue and vital bone [63]. Stubinger et al. (2010) [60] advocated and demonstrated the use of a piezoelectric device for use in sheep osseous surgery. The authors claimed excellent clinical handling and no evidence of thermal damage to the osteotomy site. The same group investigated the use of a piezoelectric device to prepare the osteotomy site prior to dental implant placement in the iliac bone of sheep [64]. Significantly, removal torques were lower in piezoelectric osteotomy site, compared to conventionally prepared sites at six weeks, but the situation was reversed at eight weeks. The results indicate different healing patterns after piezoelectric osteotomy [64]. The authors suggested that use of a piezoelectric device during sinus lift procedures might be beneficial in terms of minimizing damage to surrounding tissues [64]. The SM can be damaged during augmentation procedures which may complicate and lengthen surgery duration, possibly impairing healing and lead to an increase in post-operative complications [59]. A recent report of 100 consecutive of sinus floor elevations performed using a piezo-electric device compared to a steel bur osteotomy noted a significant reduction in SM perforations from 30% to 7% [65]. Furthermore all perforations observed were reported to occur subsequent to the use of hand instruments and not attributable to the use of the piezo-electric device [64]. The potential benefit on healing times following osseous surgery [63] may impact on comparability between studies, particularly at early healing time points.
1.2.6. Summary
The consequences of dental extraction in the posterior maxilla often include atrophy of the residual ridge and increased pneumatisation of the maxillary sinus space. Either of these events may be sufficient to reduce the amount of bone available for predictable implant placement and thus necessitate sinus manipulation or grafting to improve dental implant outcomes. There are a number of surgical approaches to the maxillary sinus depending on the clinicians experience and patient’s individual needs. Lateral sinus grafting is considered to be the technique of choice where there is insufficient residual alveolar bone to obtain primary stability. Use of piezo-electric devices is increasing as clinicians attempt to reduce risk and improve bony healing of surgical sites.

1.3. Implants in the grafted sinus

1.3.1. Survival of implants in augmented sinus sites
Analyses of the clinical survival rates of implants into augmented sinus sites are reportedly similar to those that did not require grafting \[^{2, 9}\]. Survival rates varied for different materials, but were broadly similar across all groups. For sinus sites grafted with autogenous bone, survival was reported to beat 90% \[^{2}\] and 92% \[^{9}\]. The rates were similar for implants placed into xenograft augmented sinus sites. Aghaloo et al. (2007) reported mean a survival rate of 95% over a range of follow up periods \[^{9}\] and 87% in the single study included by Tong et al. (1998) \[^{2}\]. However the number of studies using xenograft augmentation materials available for comparison was cited as being too small to make firm conclusions \[^{1, 9}\] and only one study using xenograft alone was included in the analysis by Tong et al. (1998) \[^{2}\]. The study periods varied considerably, from five to seventy four months \[^{9}\], further limiting validity of comparisons.

1.3.2. Delayed versus immediate placement
Different surgeons have different protocols, although two stage surgery is clearly more expensive and uncomfortable to the patient. The original protocols for sinus grafting as a pre-implant procedure describe a two stage technique \[^{12, 39}\]. Lack of primary stability \[^{66}\], is often cited for the use of a delayed placement protocol \[^{67, 68}\], and the report of The Academy of Osseointegration Sinus Consensus Conference (Failure Analysis Section) \[^{36}\] was that a single stage approach was
‘more prone to implant loss than a delayed technique’ \cite{16} when less than two mm of residual bone remained.

1.3.3. Influence of implants on healing of grafted sites

The presence of an implant in the region of the elevated sinus lining has been shown to affect healing significantly, although the effect is not consistent and appears to be dependent upon implant design and surface morphology \cite{16}

The question of the influence of implant macro-design was addressed by Boyne et al. (1993) in a primate model \cite{52}. The authors found clear differences in healing around different thread and apical configurations of identical TPS implants in this model. Little bone formed at the apical end of open ended and deep threaded implants, while significant bone formed spontaneously around otherwise identical round ended implants. The depth of penetration into the sinus also affected healing, in that more bone formed around round headed implants penetrating two to three millimeters (mm) into the sinus as opposed to implants that penetrated up to five mm.

With respect to the effect of surface design, systematic reviews have demonstrated that roughened surface implants have significantly improved success rates in grafted human sinuses \cite{69, 70} although the authors commented that the heterogeneity of the studies included precluded further inferences.

1.3.4. Summary

These results indicate a significant influence of implant design in the healing of grafted sinus sites. For this reason studies of healing of BO, where implants were placed simultaneously, must be interpreted with extreme caution. The need to simplify the nature of investigations into sinus augmentation materials has been repeated called for by respected authors in the field \cite{1, 16}. 
1.4. Animal Models

The use of an appropriate animal model is important for the development of clinical techniques and bio-materials. The specific animal chosen will depend on many factors, with compromises being made according to the specific circumstances of the study. A recent review examined the most significant animal models used in dental implant related research \(^{[71]}\), i.e., dogs, sheep, goats and pigs, although no one animal was found to be superior for study, the authors recommended consideration of bone density, architecture and remodeling be taken into account. A similar conclusion was reached in a comprehensive book on the subject of animal models in orthopaedic research \(^{[72]}\). The canine model has been extended to investigate sinus augmentation \(^{[73, 74]}\), however the canine maxillary sinus model has been criticized, principally due to the absence of an anatomical equivalent of the human SM \(^{[53]}\). Furthermore there is increasing concern regarding the use of companion animals for bio-medical research in many countries \(^{[72]}\). There have been no comparative studies of the suitability of one particular animal over another in the field of maxillary sinus surgery, although Haas et al. (1998) \(^{[53]}\) commented that results obtained in sheep were similar to those observed by Boyne et al. (1980) in dogs and rhesus monkeys \(^{[6]}\).

The relative merits of various characteristics of animal sourced bone to human bone are indicated in Table 1. Three stars indicate a particular characteristic is most similar to human bone, while one star indicates the bone is the least similar to humans amongst the animals compared.

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* = least similar to human, *** = most similar to human (adapted from Pearce et al. 2007) \(^{[71]}\)

The suitability of available animal models for maxillary sinus investigations are summarized next section.
1.4.1. Large animals
Estaca et al. (2008) [75], investigated the suitability of three large, adult animal species for simulation of a modified Caudwell-Luc procedure as might be used to augment the maxillary sinus. The authors compared the healthy recently euthanized heads of four pigs, four goats and four sheep, using three dimensional (3D) imaging and hands on surgical exposure of the antrum wall. All animals presented minor difficulty, in that the mouth opening was too small to allow unmodified intra-oral access. In the sheep and goats this necessitated a relieving incision from the corner of the mouth of approximately two centimeters caudally to expose the buccal vestibule, while in pigs this was even more radical – involving the further detachment of the masseter muscle. The large incision from the corner of the mouth may be considered unacceptable for live animals, in terms of their welfare. The development of extra-oral approaches to the sinus in sheep [53], goats [76] and mini-pigs [77] provides an alternative approach. The goat model was also noted as being highly suited to sinus elevation and implant studies in a recent paper [78]. The anatomy and bone density of sheep and goats, as well as their availability and cost appears to be the most favorable of non-primate species so far investigated for this purpose.

Surgical access in the pig model was restricted by the density of the cortical bone which was very thick [75], which restricted this animal’s utility for surgical simulation. Never-the-less the pig, and in particular mini-pigs, have been regularly used for maxillary sinus investigations owing to favorable handling and healing properties. Furthermore important characteristics of porcine bone are broadly similar to human bone [71,72] (Table 1).

In New Zealand the domestic sheep is an abundant animal, available in large genetically homogenous flocks. Furthermore, there is considerable experience in handling this species as well as expertise in the use of sheep for periodontal and implant research [79].

1.4.1.1 Dogs
The canine model is one of the most frequently used in dental research [71]. Dogs are relatively easy to handle and their bone has a similar composition to that of humans [80]. There is variability between bony sites however, as well as between animals of different size and age [71]. With respect to sinus surgery, there is no tissue analogous to the human SM in the canine maxillary sinus [73], limiting the utility of this model.
1.4.1.2. Pigs
Several porcine models for maxillary sinus surgery, utilizing domesticated animals and ‘mini’-pigs, have been published [81-84]. The domestic pig is similar in size to humans, although the intraoral approach to the sinus is not possible, and the site is protected by a buttress of heavy bone [75]. The domestic pig also benefits from a sinus cavity of sufficient size to allow significant elevation of the membrane, similar to that required in humans prior to implant placement [84]. These animals are large and powerful, making handling potentially hazardous [75].

1.4.1.3. Sheep
The use of sheep for orthopaedic research has been increasing [85], most likely due to public perception of this animal as being a utility breed when compared to the companion status afforded dogs [71]. Sheep are more similar in size to humans as compared to most dog species, allowing surgical techniques to have greater relevance in terms of technique and instrumentation than smaller animals [71], although there are significant differences in the microscopic structure and composition of ovine bone when compared to humans.

Sheep have a primary type bone structure, consisting predominantly of osteons less than 100µm in diameter, as opposed to the secondary type structure of humans [86]. The significance of this observation has been downplayed by a subsequent investigator, who noted ‘no appreciable differences’ in the healing of fractured femurs and tibia between dogs and sheep when fixed with internal plates [87]. A separate study examined the suitability of sheep for bio-material testing in cancellous bone [88], and described the model as excellent.

Importantly, in the context of this review, a number of studies in augmented sheep sinuses [89-98] have clearly demonstrated the utility of the sheep sinus model for implant research. It must be noted that it is not possible to load implants, or expose them to the oral environment [53] in a manner analogous to humans in this model.

The review by Martini et al. (2001) [85] concluded that mature sheep, no older than 4 years, which were well handled with suitable attention paid to housing and diet were a suitable model for study of bone metabolism and repair in humans.

1.4.1.4. Goats
The goat is a very similar animal to the sheep, although they are genetically distinct and cannot readily interbreed. With respect to orthopaedic research, their use has been predominantly
restricted to ligamental and cartilage studies \cite{72}, however there appears no reason why they might not be suitable for dental implant investigations \cite{71} and in particular the maxillary sinus research \cite{78}.

In the review by Pearce et al. (2007) \cite{71}, the authors considered their geographical location, with goats being more tolerant of increased humidity, and the availability of animals for study as determining factors in their use rather than an inherent superiority of sheep with respect to goats in dental research.

**1.4.2. Small animals**

The use of animals much smaller than humans is popular due to their ease of handling, lower costs, higher reproductive rates and early maturities. Smaller animals are, however, less anatomically analogous to humans and differ significantly with respect to bone metabolism.

**1.4.2.1. Rabbits**

The similarity between the rabbit sinus mucosal lining and humans as been described as ‘favourable’ \cite{99}, and rabbits are increasingly utilized for examining grafting materials in dental research \cite{100-104}. The rabbit is also one of the most widely used animals in musculo-skeletal research \cite{105}.

The small size of the animal makes the use of mini-implants, as opposed to standard commercially available root form implants, necessary when using this model \cite{71, 101}. The bone structure and metabolism also differ markedly from humans \cite{106}, making extrapolation of observations of bony healing difficult \cite{71}.

This animal has been utilised to compare novel bone substitutes, including BO, using a calvarial defect \cite{12, 107}.

**1.4.2.2. Guinea Pigs**

The guinea pig has been investigated with respect to sinus investigations and has a sinus membrane lining similar to rabbits \cite{108}. There are no reports of sinus augmentation in the model although BO has been used to fill the mastoid space \cite{109}.

**1.4.2.3. Rats**

Rats are very small animals, with a high rate of bone metabolism, although the maxillary sinus has been described as anatomically similar to humans \cite{110}. Rats are commonly used when
investigating new materials such as bone substitutes \cite{68, 111} and alveolar metabolism \cite{112, 113}. No investigation into augmentation of the maxillary sinus was found, probably due to the small size of the cavity in these animals.

1.4.3. Primates

Larger non-human primates such as baboons \cite{114} and chimpanzees \cite{115, 116} as well as smaller primates such as capuchin \cite{50, 117} and rhesus \cite{118} monkeys have been used in maxillary sinus research. These animals are closest in genetic and anatomic terms to humans, while the larger primates are very similar in size to humans. The number of primate studies is low, most likely due to cost and considerable and growing ethical concerns regarding the use of these animals \cite{119, 72}. This is likely to compromise reproducibility of results in the future. Histological investigations of BO in the augmented maxillary sinus of chimpanzees were reported in the late 1990’s \cite{115, 116} successfully utilizing this model. There have been no subsequent reports using chimpanzees for maxillary sinus grafting, although a limited number of reports have detailed investigations using smaller primate species such as capuchin monkeys \cite{50, 117}.

The study by Hurzeler et al. (1997) \cite{118}, is noteworthy for a number of reasons. The authors compared the response of implants placed into grafted sites in either a one or two stage technique following extractions and osseous re-contouring three months or seven months previously. Furthermore, the implants were functionally loaded for six months. The ability to study these parameters demonstrates the utility of the similar anatomy of primates for such clinical investigations. This study will be discussed again in detail in section 1.10.2.

1.4.4. Validity of animal models

No animal model will be 100% analogous to human biology. Only by careful selection of the particular animal, and especially the specific nature of the site to be investigated, can their use be justified. Continued use of a particular model relies on reproducibility of results and availability of genetically homogenous animals.

1.4.5. Summary

There is a large array of animals, which may be used to test specific hypotheses. With respect to dental implants and sinus grafting however, the larger animals appear more suited on the basis of the similar anatomical size of the desired model.
With respect to sinus surgery, the presence of tissue analogous to the SM in the sinus is desirable. The use of non-human primates apart, sheep are a highly suitable animal for study of maxillary sinus grafting. Histological evidence from the sheep sinus model was considered comparable to the mini-pig and superior to the dog model in the discussion by Haas et al. (1998a)\(^{[53]}\), while the surgical access was simpler.

There is a growing body of work reporting on the ovine sinus model, which is especially relevant to New Zealand based researchers where experience in handling and availability of homogenous flocks for subject selection is combined with a low cost for the purchase of study animals.
1.5. Bone Biology

1.5.1. Normal bone

The primary cell types of bone are osteoblasts and osteoclasts, which produce and resorb bone respectively. Osteocytes, which lie embedded in mature bone, and bone lining cells on the external surface of bone, represent different stages of differentiation of the osteoblastic cell type, depending on location and function. Bone is a dynamic and crucial connective tissue, constantly modeled and remodeled depending on the demands placed upon it. Bone modeling is the response of bone to stress and strain, involving change of shape of the tissue, while remodeling is the ongoing process of renewal and replacement with no change in architecture. During remodeling bone formation and resorption are linked in a coupled sequence whereas they occur independently of each other during bone modeling. The coupled sequence of bone remodeling is also known as the Activation-Resorption-Formation (ARF) cycle. Osteoblasts derive from mesenchymal progenitor cells in bone marrow. Their differentiation into osteoblasts, capable of forming bone, is governed by a series of transcription factors, of which RUNX2 and osterix (Osx), also known as Sp 7, are considered to be master switches. RUNX2 is a member of the RUNX family of transcription factors, which contain the runt DNA binding domain. Transcription factors interact with specific DNA sequences, enabling regulation of target gene expression. RUNX2 is also known as core-binding factor α-1 (Cbfa1), CCD, AML3, CCD1, OSF2, PEA2aA, PEBP2A1, PEBP2A2, PEBP2aA, PEBP2aA1, MGC120022 or MGC120023. RUNX2 is a master regulatory switch of osteoblast differentiation via mediation of the temporal activation and/or restriction of cellular proliferation and phenotypic genes. Interactions between the canonical Wnt signaling pathway and OSX play a major role in activation of the RUNX2, in terms of osteoblast differentiation. RUNX2 is a target molecule for TGF-β and BMP-2, via the Smad signaling pathway to the nucleus. These signals promote activation of genes associated with osteogenesis. Further interactions involving RUNX proteins, and the Sox, PPAR2 or Dlx-5 transcription factors which regulate differentiation of chondrocytes, adipocytes and myocytes respectively (Figure 1. N.B. myocyte regulation not shown).
RUNX2 appears to be involved in not just differentiation but also in some aspects of function in osteoblasts\textsuperscript{[131]}. While RUNX2 regulates the expression of bone matrix producing genes during osteoblast differentiation, it is not required for the maintenance of gene expression in mature osteoblasts\textsuperscript{[125]}. A complete understanding of the role of RUNX2 in humans has yet to be elucidated\textsuperscript{[132]}, although it has been associated with skeletal development\textsuperscript{[133]}, cartilage formation\textsuperscript{[132]} and bone remodeling\textsuperscript{[134, 135]}.

Osteoclasts are specialized, blood derived macrophages with tissue-specific bone resorbing properties. They derive from monocyte precursor cells on or close to the bone margins. They form by the amalgamation of macrophages under the stimulation of macrophage colony stimulating factor (M-CSF) and activation of nuclear factor (NF)-κB (RANK) receptors by the RANK ligand (RANKL), produced by stromal cells such as osteoblasts\textsuperscript{[136]}.

Binding of RANK and RANKL induces osteoclast differentiation, while osteoprotegrin (OPG) is a protein produced by osteoblasts and some fibroblasts that acts as a decoy receptor for RANKL,
thus allowing control of the resorptive activity of osteoclasts by inhibiting osteoclast differentiation and activation\textsuperscript{137}.

Regulation of the RANKL: OPG ratio is principally via parathyroid hormone (PTH), which can have different effects on RANKL expression by osteoblasts depending on whether it is administered continuously or intermittently\textsuperscript{138}, underlining the complexity of the osteoclast / osteoblast interaction\textsuperscript{122}. Skeletal bone is also intimately involved in important metabolic systems such as the control of serum calcium levels\textsuperscript{139}. The cytokine interactions occurring during the ARF cycle and their effects on osteoblasts and osteoclasts maintain healthy bone. Turnover of skeletal bone is estimated at approximately 0.7% per day\textsuperscript{140}, although this is likely to vary considerably amongst individuals depending on factors such as health, activity and age\textsuperscript{141}. The rate of remodeling also differs between species\textsuperscript{80}, a factor which must be taken in to account when examining evidence from animal models\textsuperscript{71}.

1.5.2. Healing bone

Healing of bony sites is characterized by complex cascades, driven by pro-inflammatory cytokines, members of the transforming growth factor β (TGF-β) superfamily and angiogenic factors\textsuperscript{142}.

Repair of bony sites is initiated by haemorrhage and platelet aggregation. The degranulating platelets release numerous cytokines including TGF-β, vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF). These cytokines induce migration of macrophages, inflammatory cells and differentiation of osteoclasts which further release cytokines such as TGF-β, (TNF-α) and bone morphogenic proteins (BMPs)\textsuperscript{143}.

Pro-inflammatory cytokines, such as interleukin-1, interleukin-6 and tumour necrosis factor-α (TNF-α) are initially released by macrophages, inflammatory cells and mesenchymal cells resident in the periosteum\textsuperscript{144}. These cytokines are involved in numerous cellular activities including the stimulation of angiogenesis and extra-cellular matrix synthesis, as well as the induction of fibrogenic cells within the healing site\textsuperscript{144}. TNF-α is also involved in the recruitment of mesenchymal stem cells and the initiation of osteoclastic activity\textsuperscript{142}.

The TGF superfamily is a large group of proteins including bone morphogenic proteins (BMPs) excluding BMP 1, TGF-β and other growth differentiating factors\textsuperscript{142}. BMPs form a large sub-group in this superfamily, binding to type-II serine/kinase receptors involved in the
transphosphorylation of type-I receptors \cite{145}, and thus initiating the Smad cascade. Smad proteins transduce signals from the TGF-β receptors to the nucleus \cite{145}. Each BMP has a different role in the repair of bone and they are expressed in a well defined temporal sequence \cite{142}. BMPs are further involved in stimulating the production of other growth factors such as insulin like growth factor (IGF) and VEGF.

The role of TGF-β is complex. It is released by platelets, secreted by osteoblasts and is also stored in the bony matrix \cite{143}. TGF-β induces production, migration and proliferation of mesenchymal stem cells (MSC’s), pre-osteoblasts and osteoblasts, amongst others \cite{143}. TGF-β also induces synthesis of essential bone proteins such as Type I collagen, osteopontin (OP), osteonectin (ON) and alkaline phosphatase (ALP) \cite{146}. Furthermore TGF-β may have a role in the inhibition of osteoclastic activity and promotion of osteoclastic apoptosis \cite{146}.

New or woven bone (WB) is relatively disorganized in structure, with multiple irregular osteocytes distributed throughout the calcified matrix. The collagen fibres traversing the WB interweave and the matrix has a high density of mineral content \cite{137}. As the bone matures, concentric layers or lamellae, approximately 3 - 5 µm across are distinguishable \cite{137}. The collagen of lamellar bone (LB) runs in parallel formation giving great structural strength to the tissue. The bone continues to mature, forming distinctive bone units, ‘osteons’ or ‘haversian systems’ with interconnecting canals between the enclosed osteocytes providing a means of nutrient flow and waste disposal, as well as a system for cell-to-cell signaling \cite{137}. Signaling between mature osteocytes may provide mature bone a means of responding to stress and strain, and thus the ability to optimise bone modeling according to local needs.

1.5.3. Graft consolidation

The complex process whereby the grafted material is incorporated into the surrounding natural bone has been described as consolidation \cite{16} and is analogous to healing bone with the addition of a graft material. This process includes the in-growth of new vasculature (angiogenesis), bone deposition (by osteoblasts), and remodeling or resorption of the integrated graft material and surrounding bone, by osteoclasts (Figure 2). Graft consolidation is an absolute necessity for successful integration of subsequently placed dental implants \cite{16}. Consolidation of graft materials will differ in regard to the dynamics of integration and incorporation of the graft particles, as well as the ability of the host to resorb the particles as part of the normal process of modeling and
remodeling of vital bone. The nature of graft consolidation will depend on the surrounding bone and the biological properties of the graft materials themselves \[16\].

**Figure 2** Overview of events surrounding cellular differentiation during graft consolidation
Adapted from Jensen (2006) \[16\]. Cellular events characterized in the proposed study are highlighted in red.

### 1.5.4. Osteogenesis

Osteogenic materials contain viable bone forming cells, osteoblasts or multi-potent cells capable of differentiating into osteoblasts, although these cells require nutrients and growth factors to function and produce bone. Osteogenic materials used in clinical practice are limited to autografts due to host rejection and disease transmission issues. Only a limited number of osteogenic cells can be transplanted and more osteoblasts need to be recruited to the site. The origin of osteogenic cells has not been confirmed, but it is proposed as peri-vascular \[147\] or circulatory \[148\] cells, indicating the importance of angiogenesis in the consolidation process.

### 1.5.5. Osteoinduction

Osteoinduction describes the process whereby osteogenesis is induced and is an important component of normal bone healing \[149\]. Osteoinductive materials induce the differentiation and migration of osteogenic cells into the grafted region. Osteoinductive agents, such as BMPs \[150\] and platelet rich plasma (PRP) \[151\] have been added to BO in an attempt to enhance bone
formation within grafts. It is possible that the surface morphology of some graft materials may also behave in an osteoinductive manner via bio-mimicry or favourable geometric morphology, although the processes are not yet well understood.

1.5.6. Osteoconduction

Osteoconductive materials do not induce osteogenic cells into the graft, but are said to provide a ‘scaffold’ which allows faster and organized consolidation of the grafted site. The boundary between an osteoconductive and osteoinductive material may become blurred if a material is highly osteoconductive. It is also possible that materials may exhibit combinations of these properties. Elucidating which is more important or prevalent is probably only achievable using techniques that analyse genetic regulation or detect the presence of proteins characteristic of defined physiological pathways.

1.5.7. Angiogenesis

The in-growth of a functioning vasculature is an important early stage in the graft consolidation process. This process is initiated by the expression of angiogenic chemokines released by platelets and later in the healing process by macrophages. In bony tissue, the osteoclasts, bone-specific macrophages and osteoblasts all have a role in modulating angiogenesis. Furthermore, it has been shown that vascular endothelial growth factor (VEGF), an essential cytokine in angiogenesis, increases the bone resorbing activity of osteoclasts. Angiogenesis appears to be inextricably linked to osteoblastic and osteoclastic activity.

1.5.8. Gradient of consolidation

The nature of graft consolidation is determined by the biology of the surrounding tissues and the bio-activity of the graft material itself, i.e., whether the material is osteoconductive, osteoinductive or osteogenic. In the case of osteoconductive materials it would be expected that the earliest consolidation would be in those regions closest to the surrounding vital bone. This dynamic has been reported in the animal BO sinus graft model. The authors described a gradient of consolidation across three regions of the grafted site. The area closest to the underlying bone was the first to be bridged by new bone growing out from the pre-existing bone. This was followed by osteoconduction into the centre of the graft material, where a vein of vital bone ‘jumped’ between particles but was ultimately connected to the bone growing in from the first zone. Finally the periphery of the graft, from the edge of the region of new bone to the area

23
directly under the elevated sinus membrane, defined the maximal distance of new bone in the
time frame reported.

1.5.9. Resorption
Depending on the graft material, resorption appears to occur via two mechanisms. The material
may be resorbed by osteoclasts, in the same manner as for calcified tissues in the body. Materials
that are resorbed by osteoclasts include autogenous grafts, allograft and some alloplast material
[^159]. All contain crystalline forms of hydroxyapatite. A second mechanism describes dissolution
in the extra-cellular matrix surrounding graft particles, such as appears to occur with β-TCP[^160],
although the authors speculated upon a minor role for osteoclasts in this study.

Some materials appear not to resorb significantly at all, and in fact have been produced in such a
way as to minimize resorption, e.g. Endabon®. Non-resorbing graft materials retain the gross
morphology of the grafted area, but the dynamics of remodeling and modeling of the graft area
may continue to be altered by the persistent graft particles. The graft area does not behave in an
identical manner to natural bone, although it appears to have utility when used as a site for
implant placement[^161].
1.6. Sinus grafting materials

Bone grafting materials can be grouped into different categories based on their source or their biological behaviour. With respect to source, the graft material can be harvested from the same animal as the recipient, i.e., auto- and allo graft. Graft material sourced from another animal is termed xenograft and synthetic, or non-animal derived material described as alloplastic. With respect to biological behaviour, the materials may be osteogenic, osteoinductive or osteoconductive.

1.6.1. Autografts
Bone graft material taken from another site in the same individual, is often referred to as the gold standard in bone augmentation. Autogenous bone can be obtained from various surgical sites, e.g., the hip, calvarium or jaw. The harvested bone may be cancellous, cortical or mixed morphology and in block or particulate form. The advantages of autogenous bone include the limited risk of disease transmission during surgery, non-antigenic nature of the graft and the transplantation of osteogenic and osteoinductive agents such as osteoblasts and Bone Morphogenic Proteins (BMPs) [162]. The principle disadvantage of autogenous bone is the need for a second surgical site, and the subsequent increase in post-operative morbidity. The different nature of the bone obtained from the varied sites used for autogenous grafts gives each a different outcome, although all autogenous material is remodeled regardless of its origin, with the denser cortical material being remodeled more slowly than cancellous-bone derived grafts [163]. Autogenous grafts are also reported to undergo considerable and unpredictable volumetric resorption [1]. Resorption of pure autogenous grafts may limit their use in long-term sinus augmentation [9], although the clinical significance of this following implant integration is not known. Additionally there may be benefits in combining autogenous bone with other graft materials in an attempt to improve healing, reduce contraction and initiate osseo-induction. Mixed grafts for sinus elevation are commonly reported [164, 165], although the ratios used are not consistent [166] making comparisons between reported mixes difficult.

1.6.2. Allografts
Allogenic bone, i.e., bone taken from a human source, is available in a number of forms such as putty, particulate and demineralized. Fresh frozen allograft bone is seldom used due to disease
transmission concerns \(^{[11]}\). Processing of allogenic bone, e.g., by irradiation and/or freezing, eliminates osteogenic potential \(^{[167]}\).

Allogenic bone may be sourced from cortical or cancellous sites which affects its behaviour following grafting. Cancellous allografts are considered osteoinductive, while cortical allografts are not \(^{[167]}\). Both cortical and cancellous allografts are considered osteoconductive \(^{[167]}\).

A further feature of allografts is variation in osteoinductive properties due to variations in donor pharmacological status, physiology and age \(^{[168, 169]}\). Variation in biological behaviour limits predictability when using allogenic bone grafts.

Allografts can be expensive, due to the degree of regulation and verification involved in sourcing the material, although the relative costs and availability of different bio-materials varies between countries \(^{[11]}\). Finally, the use of material from another person may be unacceptable to some individuals.

1.6.3. Alloplastic (synthetic) materials

There are a number of commercially available synthetic materials for bone grafting.

Tri-calcium phosphate (TCP), commonly available in the ‘β’ crystal form which is manufactured at lower temperature than the ‘α’ form and has been used as a bio-compatible filler for a number of years \(^{[170]}\). TCP is rapidly resorbed by the body and has been reported to permit bony ingrowth, however other studies have demonstrated fibrous encapsulation of the particles \(^{[171, 172]}\). The handling properties of the material have also been found to be problematic, with the material being difficult to retain at the graft site, possibly due to small particle size \(^{[173]}\).

Synthetic hydroxyapatite (HA) cones have been used as ridge preservers, before dental implants were in common use. They have been observed to slow ridge resorption \(^{[174]}\) when used as a pre-prosthetic procedure for maintaining denture stability; however the material is dense and replaced slowly. Synthetic hydroxyapatite cones are unlikely to be useful as a pre-implant procedure due to the small amount of bone available, when the site is healed, for integration and the high rate of post-operative failure necessitating cone removal \(^{[175]}\).

Two commercial ‘bio-active glass’ products, Perioglas\(^{®}\) (Block Drug Co., Jersey City, NJ) and Biogran\(^{®}\) (Orthovita, Malvern, PA), have been developed and are still currently available. They are composed of silico-phosphate chains, as used in dental restorative glass ionomers \(^{[176]}\).
Some studies have reported modest benefits when used for ridge preservation \cite{177, 178}, however there appears to be a limited in-growth of new bone amongst the particles and a long healing time \cite{179}. While this delayed healing appeared to have a limited impact upon subsequent implant success at 2 years, the slow conduction of new bone, and replacement of grafted particles at sites grafted with bioactive glass, hardly implies advantage in the use of the material. The authors considered that integration was most likely achieved at the portion of the implant not in the grafted socket, i.e., apical to the extraction site \cite{179}.

Bio-active glass may be osteoconductive in human sinus studies \cite{180, 181} although the low number of published studies indicates that it is not a popular material for sinus augmentation. A comparative study in rabbit sinuses, including BO and bio-active glass, considered BO to be a superior for sinus augmentation \cite{182}.

1.6.4. Xenografts

1.6.4.1. Coral and marine-sourced calcium carbonate

Coral is a natural product derived from the exoskeleton of certain marine creatures. It is predominantly calcium phosphate, and porous in structure, similar to bone \cite{183}. The porosity of the material is variable and dependant on a number of factors. There is a minimal risk of disease transmission with the use of this material. Coral is resorbed and replaced by bone, at a rate dependant on the initial particle size, over a period of between eight and eighteen months in human tooth sockets \cite{183}. The use of coral appears to present no great advantage over other slowly resorbing products available, and the material is highly dependent upon controlling porosity size if predictability of outcome is to be gained. The harvest of this wild-sourced material may be difficult as corals are increasingly protected in marine management programmes. Other marine-sourced products, Algipore® (Dentsply, Friadent, GmbH, Germany)\cite{184-186} and C-Graft (Clinician's Preference, Golden, CO) \cite{187}, both derived from marine algae, have been reportedly used with some success in the human maxillary sinus. Interestingly the materials may be slowly resorbable \cite{186}, and replaced by vital new bone \cite{185}, although there are only a limited number of publications available for scrutiny.

1.6.4.2. Deproteinated Bovine Bone (DBB)

DBB has been used for a number of years in alveolar grafting. There are a number of commercial products in the dental market, e.g., Bio-Oss®, Endobon®, OsteoGraf/N® and Cerabone®. DBB is
normally produced using combinations of heat, organic solvents and strong chemicals, e.g., sodium hypochlorite, with the product being sterilized using gamma radiation and/or heat and pressure. The exact process is not published for each material as this is retained as proprietary information. The production process is designed to remove all organic residues from the material, as well as optimize the structure of the residual material for its intended use. The removal of organic residue is extremely important in the prevention of disease transmission \[^{188}\]. For example, it has been suggested that BO may have residual proteins which might have an osseo-inductive effect \[^{169}\], however this was refuted in a later study \[^{189}\] which found no residual protein in the material \[^{189}\]. The risk of disease transmission from the use of BO or Osteograf/N\(^\circ\) was described as negligible in a thorough analysis by Wenz et al. (2001) \[^{188}\].

1.6.5. Summary

There appears to be no perfect sinus graft material \[^{137}\]. Each surgeon and patient will have their own individual clinical, ethical and personal requirements. The varied responses of certain materials and the uncertain long-term behaviour of graft materials emphasizes the need for the development of relevant biological models and the thorough characterization of materials in these models to improve predictability of grafted site response. DBB is regularly described as a safe and useful material for sinus grafting, either alone or in the form of a mixed graft. The success of DBB material has led to the development of a variety of DBB products. Differences between these materials are discussed in the next section (1.7).

1.7. DBB products

It is probable that the different processing methodologies used to produce DBB products will impact on the biological responses observed when they are implanted in a maxillary sinus. Processing factors have been shown to influence porosity \[^{190}\], crystalinity \[^{13}\] and potential presence of residual proteins \[^{189}\].

A further feature of the use of DBB seems to be the very slow replacement of the material in grafted sites. The material may persist for many years in the maxillary sinus \[^{191}\] \[^{192}\]. However, the persistence of residual graft material does not appear to impact on the successful integration of dental implants into areas grafted with DBB. This was demonstrated in a canine model, where
bone to implant contact was similar in grafted and non-grafted sites \cite{193}. Similarly implants placed into sheep maxillary sinus sites augmented with BO \cite{90, 91, 194} were characterized by significantly higher ‘pull-out’ forces than non-grafted sites.

Slow or negligible resorption of DBB may in fact be a benefit, with respect to long term preservation of bone volume. Re-modeling subsequent to dental extraction may result in unpredictable and/or undesirable bone and soft tissue morphology, which may be delayed or prevented by the use of ridge preservation techniques such as the use of non-resorbable bio-materials \cite{195}.

1.7.1. Physico-chemical differences between materials
While materials may differ in their origin, they can also differ in the manner of processing. These processing differences can have significant effects on their physical properties, whether macroscopic or microscopic \cite{13}. Processes which are known to affect the properties of DBB are the processing temperature and pressure \cite{13}, which appear to affect crystalinity of the microstructure and porosity of the material \cite{13, 196} as well as the carbonate content \cite{197}.

1.7.2. Reduction in carbonate groups
Natural hydroxyapatite often contains carbonate groups, although the amount is highly variable \cite{197, 198}. The importance of this is not entirely clear, although in β tri-calcium phosphate grafts, increasing carbonate content is associated with decreased resorption. Tadic and co-workers attempted to predict the resorbability of fourteen different calcium phosphate bio-materials, based on their chemical composition \cite{197}. The high resorbability of BO predicted in this study was at odds with long term clinical reports \cite{199}.

1.7.3. Sintering
Sintering describes the process of heating a powder or crystal in order to change its physical properties, principally increasing the density and crystalinity of the material while reducing its porosity. The effects of this process have been demonstrated in bovine-sourced hydroxyapatite \cite{200}.

Reduction in the degree of sintering has been shown to increase resorption of graft material in a porcine mandibular model \cite{201}. This study demonstrated increased bone infill and graft resorption at 8 months using a calcium phosphate material processed at 700°C, as opposed to the same material processed at 1300°C. This effect is well known by bio-material manufacturers, and some
advertise that their material has been highly sintered to reduce the possibility of resorption following bone grafting, e.g., Endabon® (Biomet® 3i, USA) which is processed at approximately 1200°C (manufacturers data) and GenOx® (Baumer SA, Brazil), processed at approximately 950°C [13].

With respect to natural hydroxyapatite, sintering occurs when temperatures breach 700°C, and is optimized at above 1000°C [200].

1.7.4. Porosity
While many bovine xenograft products claim morphological similarity to human trabecular bone, e.g., BO, Endobon®, GenOx® and Osteograf®, micro-porosity has been demonstrated to differ significantly [196, 202]. As these materials are sourced from the same animal, variations in processing most likely account for these differences. It has been proposed that differences in porosity, and thus the specific surface area of each material would likely influence their respective biological properties [202], where increased micro-porosity would increase resorption in vivo [198].

1.7.5. Bio-Oss®
BO has been referred to as the gold standard of bone substitution [12]. BO products are available in either block form or various particle sizes, originating from cancellous or cortical bone. ‘Bio Oss® Collagen’ is a separate product, where Bio Oss is combined with a tiny amount of porcine collagen to improve handling characteristics.

BO is processed using low temperatures (approximately 300°C) and organic solvents [169]. It is claimed to be highly porous, on a macro and microscopic level, which has been confirmed by separately published analysis [196].

The particle size recommended by the manufacturer for use in sinus grafting is 1 – 2mm, however studies often fail to report particle size, or use a smaller particle size product. The reasons for this are not clear but probably relate to familiarity, availability (the smaller particles can be used in other sites) and surgeon preference.

A number of studies have noted the osseo-conductive properties of BO [191, 203], however others have also observed delayed healing in sites grafted with the material [204, 205]. Araujo et al. (2009b) [206], observed decreased reduction in alveolar width 6 months post-extraction compared
to the region of ungrafted socket sites using BO in the same dog model. The presence of BO material in the healing socket will undoubtedly interfere with the remodeling of the clot, although delayed healing may not be necessarily be an adverse outcome, if the site is stable and predictably healed. The impact of delayed healing upon implant placement is not clear, although it seems prudent to use DBB where implant placement is planned to be later than the healing period of DBB grafted sites – typically six months.

1.7.6. Moa-Bone®

1.7.6.1. History
Moa Bone® (MB) is a bovine bone product sourced from disease-free herds in the South Island of New Zealand. It is a by-product from the production of the M-Sphere®, an orbital implant used during reconstruction of an enucleated human orbit. The sphere is inserted into a pouch in the residual sclera occupying Tenon’s space. It appears that the M-Sphere® is well tolerated in humans, causing no long term inflammatory reaction, and is a market leader in the field.

1.7.6.2. Manufacturing
The MB material is manufactured in Dunedin, New Zealand. The exact nature of the process is proprietary information, as is that for BO. Briefly, however, it has been confirmed with the manufacturer that the process is as follows.

The femurs of healthy young cattle (between 6 months and 4 years) are removed from the carcasses, and all sign of meat removed. The bone is then cut to the shape of a cube, from the lower end of the femur, submerged in water and boiled in a pressure cooker for 1 hour at 121°C. The bone is removed and spun in a centrifuge at 800rpm for one minute, rinsed and spun again. This removes the majority of excess fat and protein. The bone is then boiled and spun again prior to the next stage of processing.

The bone is removed and subjected to a vacuum for one minute, followed by 24 hours immersion in 10,000 ppm chlorine bleach. The cube is then spun at 800rpm and the process repeated several times, until the total time of exposure to the chlorine solution has reached 120 hours, at which point the cube is rinsed in water. This bleaching process is repeated a further three times before the residual material removed and shaped into the required sphere, and packaged for final sterilization.
Finally, the packaged sphere is autoclaved in a hospital grade vacuum autoclave at 134°C for just over 18 minutes, and sealed.

MB is derived from the powder that is residual to the shaping of the spheres, although for the purposes of this investigation the M-Spheres® themselves were crushed. This allowed the investigators to match the particle size of the MB and BO, which would not be possible with the finer dust which the current milling process creates. The principal physical observation concerning the nature of MB and the M-Sphere® is that the material is very light, porous and friable [207, 208].

Two histological reports are available for the M-Sphere® [207, 209], neither of which concern the grafting of bony sites. Six recovered M-Spheres® were examined histologically, following their removal from restored human orbits [210]. Five of these were failing implants which the authors removed due to infection within two weeks of placement. The final case concerned a recovered post mortem specimen obtained after 10 years of healing. The authors described a mature fibrovascular tissue throughout the implant. They also noted the presence of occasional giant cells and very small areas of dystrophic calcification [210]. When the M-Sphere® was placed into the eye socket of rabbits, following enucleation of the globe, a fibrovascular infiltration was observed with no adverse pathological reaction noted. The M-Sphere® was considered to be similar to other orbital reconstruction prosthesis’ available [207, 209].

1.7.6.3. Quality assurance

The M-Sphere® is regulated as a human medical device in many countries / regions, including New Zealand, Australia, United States of America and Europe. There have been no safety concerns regarding the use of the M-Sphere®, and it has received continued approval from regulators during the periods of heightened concern with respect to bovine-sourced products and prion-related diseases.
1.8. Use of membranes to close the osteotomy site following sinus grafting

The use of barrier membranes has found many surgical applications. In particular they are useful for excluding certain cell types during the healing phase following surgery, as described by Gottlow \cite{211} and Nyman \cite{212}. In periodontal and oral surgical literature this is known as Guided Tissue Regeneration (GTR) \cite{213}, and has a sound scientific basis. The underlying principal is that some tissues, such as fibrous connective tissue and epithelium, tend to invade a wound at a faster rate than slower proliferating tissues such as bone \cite{214}.

With respect to coverage of the window defect created during lateral sinus lift procedures, the evidence from a systematic review is strongly in favour of the use of barrier membranes \cite{69}. The membrane used to close the osteotomy site created during maxillary sinus grafting has two main functions. Firstly, it will help contain the graft in the augmented site and secondly, it will prevent in-growth of soft tissue from the tissues overlying the bone into the healing graft site. Graft containment appears to be adequately achieved by careful repositioning of the soft tissue flaps \cite{215}, however prevention of soft tissue in-growth has been shown to positively affect survival of implants placed in augmented sinuses \cite{215}.

1.8.1 Human studies

Histological information from human subjects is limited, due to the narrow scope of the standard trephine biopsy. One study has compared the differences in bone formation, using a split mouth design, in a randomized comparison between membrane (e-PTFE) and non-membrane protected lateral window defects \cite{215}. The authors found very significant differences and drew clear conclusions, within the scope of a small sample size of 12 participants. The amount of bone formed in unprotected defects was less than half of the amount observed in membrane protected sites. The authors went on to demonstrate the positive influence this had on implant survival and recommended that barrier membranes be used in all lateral wall access sinus graft procedures \cite{215}.

1.8.2. Animal studies

McAllister et al. (1998) \cite{216}, clearly showed in-growth of soft tissue into unprotected lateral window defects in chimpanzees following a healing period of nearly eight months. The authors commented that the use of BO in this model decreased the amount of soft tissue ingress and this may obviate the need for membrane placement.
1.8.3. Summary
The benefits of closing the window in the sinus antral wall with a membrane following lateral sinus augmentation have been investigated sufficient times for the technique to be considered mandatory[^215]. Furthermore, membranes are a reliable biomaterial utilized in sinus surgery to repair tears or perforations in the SM prior to site closure[^217]. The technique of using BO and the resorbable Bio-Gide® collagen membrane to augment the sinus could fairly be described as a standard procedure.
### 1.9. Analytical techniques for osseous healing

There are a large number of techniques used to analyse hard tissue healing. The benefits and drawbacks of commonly used techniques are summarised in Table 2.

**Table 2 Summary of commonly used techniques for analysis of healing osseous grafts**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Observed Parameters</th>
<th>Strengths</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology (decalcified paraffin-embedded sections)</td>
<td>cell morphology</td>
<td>commonly employed, simple techniques, readily reproducible</td>
<td>processing artifacts, positive identification of cell types impossible</td>
</tr>
<tr>
<td>Histology (resin-embedded sections)</td>
<td>cell morphology</td>
<td>commonly employed, simple techniques, readily reproducible</td>
<td>positive identification of cell types impossible</td>
</tr>
<tr>
<td>Histochemistry / Immunohistochemistry</td>
<td>proteins and cellular markers</td>
<td>positive identification of cells and cellular activities</td>
<td>lack of specific antibodies, complicated processing, false positive and background staining</td>
</tr>
<tr>
<td>Radiography</td>
<td>mineral density</td>
<td>simple, cheap, non-invasive</td>
<td>limited quantity of information</td>
</tr>
<tr>
<td>Micro-computed Tomography (µCT)</td>
<td>mineral density (3D)</td>
<td>3D imaging of healing site</td>
<td>Segmentation: difficult to separate graft material from new and pre-existing bone</td>
</tr>
<tr>
<td>Polymerase Chain Reaction (PCR)</td>
<td>gene expression</td>
<td>precise targeting of genes of interest</td>
<td>expensive, need for specific primers for target genes, importance of individual gene expression can be overestimated or misinterpreted</td>
</tr>
</tbody>
</table>
1.9.1. Histological, histochemical and immunohistochemical tools

There are a number of immunohistochemical tools available with which to examine bony healing. Useful antibodies correlate with important cellular processes known to occur during graft consolidation such as, but not limited to, proliferation, osteogenesis, apoptosis, calcification and vascularisation.

1.9.2. Osteoblasts

1.9.2.1. RUNX2

The identification of osteoblasts on morphological grounds alone, in the absence of immunohistochemical staining, cannot be considered accurate \[^{218}\]. Immunohistochemical identification of osteoblasts is often made using antibodies to identify proteins such as ALP, OPN, ON and type I collagen. These and other proteins which are typical of osteoblast function may not be present at all stages in the development and function of the osteoblasts \[^{125}\] (Figure 3). Characterization of these stages when analyzing bone healing has been noted as being necessary but problematic \[^{218}\]. RUNX2 has been used in recent immunohistochemical investigations of sinus grafts \[^{100,159}\], due to its’ ability to label osteoblasts at all stages of differentiation except the mature osteocyte \[^{125}\]. RUNX2 proteins can be detected in the cytoplasm or the nucleus of cells, depending on the functional stage or context of the differentiating osteoblast \[^{219}\]. As with our knowledge of other aspects of RUNX2 metabolism, the temporal distribution of the protein between the various compartments of the cell is currently poorly understood \[^{219}\]. RUNX2 has been described as the ‘earliest and most specific marker for osteogenesis’ \[^{220}\], which emphasizes the utility of RUNX2 as a marker for osteoblasts at all stages of differentiation and function except maturity. Pre-osteoblasts in fibrous connective tissue, mature osteoblasts on the surface of new bone and immature osteocytes in new bone have all been demonstrated to stain positively for RUNX2 in human sinus tissue \[^{160}\]. In contrast, mature osteocytes, embedded in lamellar bone, did not stain positively for RUNX2 in the same study \[^{160}\].
1.9.2.2. Other osteoblastic markers

There are several other cellular markers commonly used to identify osteoblastic activity in decalcified, paraffin-embedded tissue. These include ON, OP, bone sialoprotein (BSP), osteocalcin (OC), BMPs and ALP. These markers are only found at specific stages of osteoblastic differentiation or function. Furthermore, detection of these proteins has been described as quite variable, as is species cross reactivity of available antibodies which can significantly affect positive identification of osteoblasts in bone specimens. Detection of OSP and BSP are usually only possible during the early stages of osteoblastic function, and OC only found in mature osteoblast and osteocytes.

Two studies have used RUNX2 labeling in addition to one or more of these more specific markers of osteoblastic function to identify osteoblasts in studies of healing human sinus grafts. In both cases RUNX2 staining was more abundant in the cells of the healing graft than other osteoblastic markers used. The authors speculated that RUNX2 positive cells would mature and deposit more bone with time. The positive staining of other osteoblastic markers was interpreted as being indicative of osteoblast function, sufficiently specific to detect distinct stages of graft consolidation.

1.9.3. Osteoclasts

Historically, osteoclasts have been identified on the basis of their behavior and location. They are the only multi-nuclear giant cell capable of resorbing bone. Active osteoclasts occupy distinctive

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**Figure 3 Relative expression of cellular markers during osteoblast differentiation**

+ = detected, ++ = detection increased relative to +

OP = osteopontin, OC = osteocalcin

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depressions in the bone surface – ‘Howship’s Lacunae’ or ‘resorption pits’. They are polarized, so that the resorptive part of the cell, the ‘ruffled border’ faces the bone.

The identification of tartrate-resistant acid phosphatase (TRAP) as a histochemical marker for osteoclasts was made by Minkin et al. (1982) [227]. TRAP staining is commonly performed to confirm identity of osteoclasts, although activity is still confirmed by the presence of resorptive areas.

TRAP staining has been used to demonstrate osteoclastic activity in healing sinus grafts [159, 160] and in healing bony sites in sheep models [228, 229]

1.9.4. Proliferation markers, Ki67 and PCNA

Cellular proliferation is an important and fundamental component of growth and repair in biological systems [230]. Two protein markers of proliferating cells have been regularly reported in the literature, proliferating cell nuclear antigen (PCNA) and Ki67, a protein encoded by the MK167 gene [231-233].

Ki67 can be detected in all active stages of cellular proliferation [234], and is completely absent amongst resting cells [233]. It has been used to detect osteoblastic and osteoclastic activity around healing grafts of BO in sheep [235]. The highest expression of Ki67 positive osteoblasts was observed at eight weeks. The principle drawback of Ki67 immunohistochemical investigation is the small number of positive cells typically identified in non-tumorous tissue [231].

PCNA aids in DNA replication during proliferation, and has been used to assess the healing events surrounding bone fractures [236] and human sinus augmentation [237]. It detects proliferative activity during the ‘S’ phase of the cell cycle [238], when cells are undergoing mitosis.

The suitability of these two markers for particular investigations is not well understood. It would appear that Ki67 is advantageous when investigating cancerous tissue, especially with respect to prognosis [231, 233]. The use of PCNA to quantify the proliferative events during bone fracture healing in a number of recent investigations [236, 239, 240] indicates the utility of this proliferative marker in otherwise healthy bony sites.
1.9.5. Other immunohistochemical markers considered for examining graft consolidation

Other significant features of graft consolidation include angiogenesis (vascular in-growth) and apoptosis (cell death). These two components associated with the healing sinus graft sites have been described in recent studies.

Degidi et al. (2006) [241] examined aspects of angiogenesis using vascular endothelial growth factor (VEGF) in specimens obtained from sinus sites grafted with BO, at three and six months. The results suggested a relationship between angiogenesis and osteogenesis in these sites.

Programmed cell death, apoptosis, is a significant component of bone maturation [242]. This feature of maturing bone was investigated in a sheep mandibular model, using the apoptotic marker Caspase 3 [235]. The authors noted elevated apoptotic activity during the later stages of graft consolidation, from sixteen weeks. They concluded that necrosis was not the fate of the majority of osteoblasts in maturing bone in agreement with the suggestion by Lynch et al. (1998) [242]. The significance of osteoblast necrosis in maturing bone is poorly understood [243].

1.9.6. Summary

There are many techniques available to enable analysis and understanding of healing in osseous grafts. The use of RUNX2, allows identification of osteoblasts in all stages of differentiation, barring maturity. This information combined with identification of active osteoclasts and proliferating cells provides a first step in the understanding of a healing graft site, prior to targeting of particular stages of osteoblast function and interaction with osteoclasts with different cellular markers at different healing time points.
1.10. Histology of Bio-Oss® in Maxillary Sinus Sites

1.10.1. Review of human reports

There are many reports available of human histology following grafting of oral sites with BO. For the purposes of this literature review, only reports of BO grafted sinus sites, in the absence of simultaneous implant placement, using a barrier membrane will be considered representative of comparable healing to the proposed study. This literature review will use some of the techniques of a systematic review in an attempt to isolate and examine only studies most relevant to the proposed investigation; however it is not intended to conform to all of the rigorous criteria of a systematic review.

There have been a limited number of published studies detailing the healing of BO in membrane-protected sinus augmentations without concomitant placement of an implant or use of a mixed graft technique, such as autogenous bone. A significant confounding factor is the considerable heterogeneity within and between studies. Variations in study parameters included the observed healing time of the graft, age of participants, inclusion of smokers and the fate of the bony window created in the antral wall.

1.10.1.1. Included studies

Thirteen studies were ascertained to be sufficiently analogous to the proposed investigation. Eight of these studies used Bio-Gide® to close the window created in the antral wall [67, 161, 241, 244-248]. The study by Degidi et al. (2007) [249], investigated two groups of ten subjects at three and six months respectively, which are reported separately for this analysis. Orsini et al. (2007) [245], discusses a case report using data from the same patient at two different time points. The two time points are analysed separately, as for the previous paper, bringing to ten the total number of included studies using Bio-Gide® to close the osteotomy site.

Two studies used the cross-linked collagen membranes, Ossix® [68] or BioMend® [250]. These membranes have been reported to degrade more slowly than Bio-Gide® in a rat soft tissue model [251]. These studies have been included in the analysis, as the primary role of the membrane is to exclude soft tissue ingress to the region containing the graft material. Furthermore, the trephine biopsy is unlikely to have been taken close to the membrane, so the noted effects on local healing
associated with this type of membrane are unlikely to have impacted on this section of BO grafted sites.

A further study \cite{252} used a polylactic acid resorbable membrane, Inion\textsuperscript{®}. This membrane material has seldom been reported in the literature, but similar healing of bone defects was reported when Inion\textsuperscript{®} was compared to Bio-Gide\textsuperscript{®} \cite{253}. The authors concluded that the only significant difference was patient preference for a synthetic product. As with the cross-linked membranes the study was included but interpreted with caution.

Parameters that were noted, but not used to exclude studies, were the stated surgical technique, i.e., whether the bony window was in-fractured or discarded, the height of residual bony ridge, inclusion of smokers in the study group, variability in the particle size of BO used and the presence of teeth proximal to the augmented region. Each of these factors may have influenced healing, however all studies differ in some respects and human studies are particularly heterogeneous due to subject and operator variables. This is a significant factor in favour of the development of animal models, where many of these parameters can be controlled more adequately.

1.10.1.2. Excluded studies

Comparing studies that used different dental implant systems is notoriously difficult, and the quality of published literature described as low \cite{254, 255}. Studies reporting histology from combined implant grafting cases have been removed from this analysis due to the variable influence of the dental implants on healing, implied by the significantly different survival rates reported in the literature \cite{16, 69, 70}. Not only does the presence of an implant alter healing characteristics of the grafted maxillary sinus, but the length of the implant might also influence healing due to tenting up of the SM \cite{48, 49, 54}. The variety of implant systems and surface structure used in these studies make interpretation of this data very difficult and potentially misleading.

Finally, the addition of autogenous bone, in varying proportions, introduces the potential for osteoinductive and/or osteogenic healing. There is conflicting evidence regarding the osteogenic potential of mixed grafts. Froum et al. (1998) \cite{256} found a substantial increase in new bone formation when mixing autogenous bone and OsteoGraf/N\textsuperscript{®}, a bovine product similar to BO. In contrast a recent paper found no significant difference in the histologic healing of a mixed graft and a pure bovine mineral (NuOss\textsuperscript{®}) graft at four months \cite{257}. Furthermore, comparison of
resorption in mixed and pure BO grafts is compromised, due to the different initial density of BO particles.

Little is known about healing of BO grafted sinus sites at early time points in humans, due to the timing of biopsy harvesting, which is nearly always performed at implant placement, to avoid unnecessary morbidity to the patient. In the absence of clarification of the benefit of addition of osteogenic material, it seems sensible to examine a simplified system without introducing additional variables such as autogenous bone or dental implants. The proposed investigation examines the use of pure grafts, without additive materials or implants, contained between the SM and the antral wall or barrier membrane. Studies that have reported histological healing in humans, and that satisfy these parameters are shown (Tables 3 and 4). Healing times reported were coincident with implant placement in all cases.
Table 3 Studies included in this review of histological reports of membrane protected maxillary sinus sites grafted with Bio-Oss®, without simultaneous implant placement in humans

<table>
<thead>
<tr>
<th>Author</th>
<th>Methodological differences and possible confounding factors</th>
<th>Histological technique</th>
<th>Other analysis</th>
<th>Smokers included</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011 Lee</td>
<td>Bony access window replaced</td>
<td>unspecified</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>2009 Kim</td>
<td>Only where insufficient bone for primary implant stability</td>
<td>DPE</td>
<td>No</td>
<td>Not stated</td>
</tr>
<tr>
<td>2009 Ferreira</td>
<td>Only where insufficient bone for primary implant stability</td>
<td>DPE</td>
<td>Radiographic</td>
<td>No</td>
</tr>
<tr>
<td>2009 Felice</td>
<td>Compared Bio-Oss® with rigid dome / barrier</td>
<td>URE</td>
<td>CT scan</td>
<td>Yes</td>
</tr>
<tr>
<td>2008 Cordaro</td>
<td>Hinge technique* / 4 trephined biopsies lost</td>
<td>URE</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2007 Orsini</td>
<td>Hinge technique* / same patient reported at 20 months and 7 years, but medically unwell</td>
<td>DPE and URE</td>
<td>TEM</td>
<td>Not stated</td>
</tr>
<tr>
<td>2007 Orsini</td>
<td>Same patient reported at 20 months and 7 years, medically unwell</td>
<td>DPE and URE</td>
<td>TEM</td>
<td>Not stated</td>
</tr>
<tr>
<td>2006 Degidi</td>
<td>Study of vascularity of grafts</td>
<td>DPE and URE</td>
<td>Immunohistochemistry</td>
<td>No</td>
</tr>
<tr>
<td>2006 Degidi</td>
<td>Study of vascularity of grafts</td>
<td>DPE and URE</td>
<td>Immunohistochemistry</td>
<td>No</td>
</tr>
<tr>
<td>2006 Lee</td>
<td>Hinge technique / Same patient group sampled at 12 months after implants placed</td>
<td>URE</td>
<td>No</td>
<td>Not stated</td>
</tr>
<tr>
<td>2006 Froum</td>
<td>Hinge technique* in some un-named cases / 2 trephines lost</td>
<td>URE</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>2005 Orsini</td>
<td>Hinge technique*</td>
<td>DPE and URE</td>
<td>SEM / TEM</td>
<td>No</td>
</tr>
<tr>
<td>2000 Yildirim</td>
<td>Hinge technique* / 10% implants lost prior to loading / biopsies of different depths according to length of implant placed</td>
<td>URE</td>
<td>No</td>
<td>Not stated</td>
</tr>
</tbody>
</table>

DPE = decalcified paraffin-embedded microtome sections, URE = undecalcified resin-embedded ground sections,* hinge technique refers to infracturing of the bony window created during the osteotomy against the intact Schneiderian membrane.
Table 4 Summary of histological findings from this review of reports of membrane protected maxillary sinus sites grafted with Bio-Oss®, without simultaneous implant placement in humans

<table>
<thead>
<tr>
<th>Author</th>
<th>n =</th>
<th>Healing Time</th>
<th>Residual BO % (mean)</th>
<th>New Bone% (mean)</th>
<th>Connective Tissue% (mean)</th>
<th>Resorption</th>
<th>Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011 Lee</td>
<td>25</td>
<td>9 months</td>
<td>40</td>
<td>19</td>
<td>41</td>
<td>‘insignificant’</td>
<td>Bio-Gide®</td>
</tr>
<tr>
<td>2009 Kim</td>
<td>5</td>
<td>4 months</td>
<td>35.6</td>
<td>NQ</td>
<td>NQ</td>
<td>not reported</td>
<td>Ossix®</td>
</tr>
<tr>
<td>2009 Ferreira</td>
<td>7</td>
<td>11.4 months</td>
<td>38</td>
<td>8</td>
<td>52</td>
<td>‘frequently observed’</td>
<td>Bio-Gide®</td>
</tr>
<tr>
<td>2009 Felice</td>
<td>10</td>
<td>6 months</td>
<td>36.1</td>
<td>33</td>
<td>31</td>
<td>not reported</td>
<td>Inion®</td>
</tr>
<tr>
<td>2008 Cordaro</td>
<td>18</td>
<td>6 to 8 months</td>
<td>19.8</td>
<td>37.7</td>
<td>42.5</td>
<td>not reported</td>
<td>Bio-Gide®</td>
</tr>
<tr>
<td>2007 Orsini</td>
<td>1</td>
<td>20 month</td>
<td>NQ</td>
<td>NQ</td>
<td>NQ</td>
<td>inconclusive</td>
<td>Bio-Gide®</td>
</tr>
<tr>
<td>2007 Orsini</td>
<td>1</td>
<td>84 months</td>
<td>NQ</td>
<td>NQ</td>
<td>NQ</td>
<td>inconclusive = ‘seemed smaller’</td>
<td>Bio-Gide®</td>
</tr>
<tr>
<td>2006 Degidi</td>
<td>10</td>
<td>3 months</td>
<td>NQ</td>
<td>NQ</td>
<td>NQ</td>
<td>‘scant giant cells’</td>
<td>Bio-Gide®</td>
</tr>
<tr>
<td>2006 Degidi</td>
<td>10</td>
<td>6 months</td>
<td>NQ</td>
<td>NQ</td>
<td>NQ</td>
<td>‘scant giant cells’</td>
<td>Bio-Gide®</td>
</tr>
<tr>
<td>2006 Lee</td>
<td>14</td>
<td>6 months</td>
<td>18</td>
<td>30</td>
<td>52</td>
<td>no osteoclasts or resorption</td>
<td>Bio-Gide®</td>
</tr>
<tr>
<td>2006 Froum</td>
<td>9</td>
<td>6 to 8 months</td>
<td>12.4</td>
<td>33</td>
<td>54.6</td>
<td>not reported</td>
<td>Bio Mend®</td>
</tr>
<tr>
<td>2005 Orsini</td>
<td>18</td>
<td>6 months</td>
<td>NQ</td>
<td>NQ</td>
<td>NQ</td>
<td>‘no signs of resorption’</td>
<td>Bio-Gide®</td>
</tr>
<tr>
<td>2000 Yildirim</td>
<td>15</td>
<td>4 to 9.5 months</td>
<td>15</td>
<td>30%</td>
<td>56%</td>
<td>‘some resorption’</td>
<td>Bio-Gide®</td>
</tr>
</tbody>
</table>

NQ = not quantified, n= number of grafts reported
Histology from all of these studies was reported using undecalcified and/or decalcified sections. Ground, undecalcified specimens were prepared in the manner similar to that described by Donath and Rohrer \(^{[258, 259]}\). Different methods were used to decalcify specimens before paraffin embedding. The majority of decalcified sections were prepared using strong acid formulations, hydrochloric acid (CalciClear Rapid\(^{TM}\), Kim et al. 2009) and nitric acid (Ferreira et al. 2009). One group used Ethylenediamine tetra-acetic acid (EDTA) at 4.13%, but did not report the length of time taken to achieve decalcification or how completion of decalcification was determined. Decalcification protocols have been demonstrated to significantly affect immunohistochemical detection of target proteins \(^{[260]}\).

1.10.1.3. Histological reports

Eight studies \(^{[67, 68, 161, 178, 244, 246, 252]}\) quantified the percentage of new bone formation, which ranged from eight to 37.7 percent of the examined volumes. In the study with the shortest time point of four months \(^{[68]}\), variability in the amount of new bone was highest and some specimens exhibited fibrous connective tissue rather than osseous healing. The amount of new bone was lowest in two of the studies that included smokers \(^{[244, 250]}\).

Residual graft material was reported but not quantified in all studies. Where residual graft material was quantified, it occupied approximately one-third of the residual volume in four studies \(^{[67, 68, 161, 252]}\). This amount correlates with the reported density of non-resorbing BO particles in recently packed defects \(^{[12, 107]}\), and indicates minimal resorption of BO in these studies \(^{[204]}\). Quantified reports of residual graft material were between 12.4 and 19.8% in the remaining papers \(^{[244, 246, 248, 250]}\). Only one of these studies claimed to observe resorption of BO particles \(^{[246]}\), although the authors failed to detect osteoclasts in the biopsies. The distribution of BO particles in grafted sites may not be homogenous, as has been described when grafted extraction sockets \(^{[204]}\). It is unclear whether the distribution of graft particles is homogenous in human maxillary sinus grafts.

The distance of graft material from the pre-existing bony sinus wall has been reported to affect healing and bone density in the grafted site \(^{[158, 261]}\). Busenlechner et al. (2009) \(^{[158]}\), described a gradient of healing around graft particles beneath the elevated SM in a porcine model, whereby the region of the graft closest to the pre-existing antral wall formed new bone earlier than regions distant from the pre-existing bone. The study by Lee et al. (2011) \(^{[161]}\) examined the possibility of a gradient in the healing pattern of human maxillary sinus grafts, whereby some regions of the
graft consolidate earlier than others. The authors noted no significant difference in the amount of new bone or graft material at nine months, whether the region sampled was directly adjacent to either the residual alveolus or the elevated sinus membrane. This would indicate that osseous healing of these grafts was near completion, although healing was still progressing as indicated by the minimal quantities of lamellar bone observed.

Summary

In general however, the results of these studies show great consistency. Residual particulate BO is readily observed in about the same amounts, in a matrix of new bone and marrow with no inflammation. The amount of new bone observed does varied significantly between studies as well within studies. This suggests that the rate of healing of grafted maxillary sinus sites is unpredictable. However, the reported survival of implants placed in these studies, greater than 90%, indicates that the biology of the grafted area is suitable for successful implant placement after six months of healing in humans. These results are similar to the findings in the review by Merkx et al. (1999), of the use of DBB material for maxillary sinus augmentation [262]. The authors noted great disparity in the methodology of the available studies, including the quality and quantity of residual alveolar bone, the implant surfaces and surgical protocols. Variability between studies regarding these factors prevented firm conclusions from being made. Significantly, they made a ‘plea’ for systematic investigations that might ascertain the ideal grafting material or mixture of materials. Additionally, they called for the use of animal models as well as clinical trials to achieve this goal.

1.10.2. Sinus grafting with BO in animal models

Animal models have been used to examine BO in a number of osseous sites [263-265]. However, no animal studies could be located that refer to the simple model proposed, i.e., healing of BO in a membrane-protected lateral sinus graft.

A number of articles were found which reported sinus grafting of BO simultaneous with implant placement [53, 81, 91, 115, 116, 118] and one of a grafted sinus without membrane protection [266]. Further studies report more complicated models, such as using graft particles as a carrier for growth factors or other bio-active compounds [77, 151].

Importantly, in the context of this study, it was claimed that BO was completely resorbed [267], or not resorbed at all [265] in different sites using the same animal model. It is likely that the
variations in the physiology of various bony sites dramatically affects resorption and thus graft behavior, indicating the relevance of developing appropriate models analogous to the human situation.

1.10.2.1. Haas et al. 1998 ‘a’ and ‘b’

The studies by Haas et al. (1998a and b) \[^{[53, 91]}\] are of particular interest in that they examine BO in the sheep maxillary sinus, albeit following one-stage surgery with concomitant implant placement. Importantly, no membrane was used to close the osteotomy site. The authors stated that this was closed only by soft tissue. The osteotomy defect was not closed at any of the time-points for either autogenous or BO test groups.

The reports relate two separate aspects of a single study that used 27 sheep. A total of 54 sinus sites were studied, evenly divided between a graft of BO, autogenous bone and no graft at all. Two implants (3.75mm x 8mm cylindrical, titanium plasma-sprayed (TPS), Friatec\(^\circledR\)) were then placed into each sinus cavity. No ‘sham’ surgery was reported in the 18 sites that were not grafted. Bone was labeled using a polyfluorochrome technique \[^{[23]}\], and the implants were unloaded. Three time points, (12, 16 and 26 weeks) were examined. Six sites were available for each test-group and time-point. Following harvest, the specimens were divided so that a single implant could be analysed separately. Specimens for histomorphometric analysis were embedded in resin, prepared and polished to approximately 20\(\mu\)m before staining with toluidine blue.

In the first paper \[^{[53]}\], histomorphometric results were reported. \(\textit{In vivo}\) staining demonstrated delayed healing in the BO group. Woven bone was clearly remodeling in the BO specimens at 26 weeks, compared to the mature lamellar bone that dominated sections in the two other groups by 16 weeks. There was a large variation between specimens in the amount of bone encapsulating the implant and BO particles, although all specimens showed bone partially encapsulating BO particles where they were adjacent to existing bone. This appears to reinforce the concept of BO being an osteoconductive material.

Interestingly, in the BO groups, the authors reported osteoclastic activity and resorptive lacunae on the graft particles at all three time points. An image of such an osteoclast was shown in the paper, although it lacks the classic polarization and ruffled border of an active osteoclast, while the lacuna no more than a shallow depression. \(\text{TRAP}\), staining for osteoclasts was performed to confirm the identity of these cells.
In the autogenous grafted group, the most notable feature was the resorption of graft material over the course of the experiment. There was more bone-to-implant contact than for either of the other two groups, but after 26 weeks, little graft material remained and only some implants had bone covering their apical extent. The authors noted the similarity between reports of resorbable sinus grafts in humans [18] and their own observations.

The lack of a sham operation raises concern that the comparisons were not truly valid where the no-graft technique was concerned. It is known that elevating the SM and preventing its collapse will result in bony infill [49] (see section 1.2.4). The round ended, TPS surface resulted in similar percentages of bone to implant contact to that previously reported in monkeys [52].

The second paper from the same study concerned pull-out tests for the alternate implant [91]. Of note in this paper is that the initial pull out strength, at 12 weeks, of BO augmented sites was significantly higher than those of the autogenous grafted and control sites. The BO and autogenous grafted sites were equivalent in the test at 26 weeks, and significantly superior to the ungrafted control. The relevance of pull out testing for implants is not well understood, being diametrically opposed to the direction of force in occlusal function. The implants in this study were a threadless design and never loaded. These aspects of the study limit extrapolation to clinical implant dentistry, however this test appears to gives a good indication of the quality of integration achieved in the sheep sinus model.

1.10.2.2. Other studies

One particular investigation of note is the work by Hurzeler et al. (1997) [118] in a rhesus macaque model. This study examined BO grafted into the sinus space above healed edentulous maxillary ridges, where the teeth had been extracted and the ridges reduced to no more than 4mm. IMZ® TPS implants were placed at the same time as sinus augmentation and additionally at four months. A further investigation was made, whereby the implants on one side of the mouth were loaded five months following the second implant placement operation. The study design was such that the implant surgeries were staggered to allow sacrifice of the animals and comparison of the various parameters in a minimal number of animals, i.e., four.

All implants were stable at sacrifice. The authors noted that the implants placed four months after grafting had more bone-to-implant contact than those placed at the time of grafting, although this was higher again in residual alveolar bone than the BO grafted region. The authors concluded
that BO used in a two-stage, delayed implant placement protocol was preferable to simultaneous grafting and implant placement. The importance of this conclusion underlines the aim of the proposed investigation, to understand the healing of grafted maxillary sinus sites in the absence of simultaneous implant placement.

Studies of sites grafted with BO report broadly similar results, especially when a GBR approach is used, in the absence of implants. In membrane-protected grafts, BO is slowly surrounded by new bone, which matures over time. Some reports describe fibrous encapsulation of the material, but this seems most likely due to failure to of the GBR technique, rather than the material.

The most significant feature of the use of BO in bony grafting is whether it is resorbed and replaced with bone over time or not, and whether this might be site dependant. The current view appears to be that the material is, at best, slowly resorbed. The need for development of simplified models to characterize healing and develop optimal surgical protocols has been stated previously [1].

1.10.3. Immunohistochemical analysis of human sinus grafts

Immunohistochemical analysis of human maxillary sinus grafts has rarely been reported. The most complete report [159] details a thorough characterization of biopsies from 12 individuals who had various oral sites grafted with NanoBone®, an alloplastic hydroxyapatite material in a silica gel matrix [268]. Three of the cases involved sinus lifts, although these were not examined separately. The study used immunohistochemical staining techniques for alkaline phosphatase, bone morphogenetic protein-2 (BMP-2), collagen type I, Ectodermal Dysplasia 1 antigen (ED1), osteocalcin, osteopontin, RUNX2 and Von-Willebrand factor to investigate osteogenesis, bone remodeling, resorption and angiogenesis. They also reported TRAP staining for osteoclasts. The authors justly claimed that this type of analysis was valuable when attempting to gauge the osteoinductive or osteoconductive properties of graft materials. The results described four stages of graft consolidation, finally resulting in mature lamellar bone. They were also observed osteoclastic activity using ED1/ TRAP and osteogenesis using RUNX2 staining [159].

Of particular relevance to our study, a recent paper reported use of a narrower range of immunohistochemical markers to analyse maxillary sinus sites grafted with β-TCP [160]. Within the context of the present study, this report validated the use of RUNX2 and TRAP for characterization of resorption and osteogenic activity. The study also examined bone sialoprotein
(BSP) and osteopontin (OPN) to confirm the activity of cells which also stained positively for RUNX2.

Only three reports were located which described immunohistochemical investigations of BO grafts \(^{[249, 269, 270]}\). These reports all related to angiogenesis and micro-vessel density, an important component of healing, but one that was not examined further in the current investigation.

### 1.10.4. Immunohistochemical analysis of osseous grafting in animal models

As for the human investigations, immunohistochemical analysis of BO grafts has seldom been reported in animal models. Studies were located that discuss angiogenesis and aspects of osteogenesis \(^{[271-273]}\) although particularly relevant to this study are those reports using RUNX2, proliferation markers, or involved sheep.

The suitability of RUNX2 for investigating sinus grafts in animals was demonstrated in rabbits \(^{[100, 274]}\). The authors found this marker particularly useful in determining the temporal effect of the graft material on bone healing when comparing different graft materials. Interestingly, the study also examined angiogenic factors, which did not differ significantly during any of the time-points studied \(^{[100]}\).

With respect to sheep, two recent reports concern the healing of membrane-protected onlay grafts in an ovine mandibular model \(^{[228, 235]}\). In these studies the investigators reported active proliferation of osteoblasts on the surface of BO particles, although definitive characterization of these cells was not performed. TRAP staining of the materials indicated active resorption of the BO, with resorption lacunae associated with positively stained cells.

### 1.10.5. Validity of studies which compare two or more materials

Three of the studies analysed in the previous section either compared BO with one or more grafting material \(^{[68, 244, 250]}\) or with others means of promoting new bone in the sinus \(^{[252]}\). For sinus grafting models, there is no possibility of using a negative control because the elevated sinus will rapidly collapse without some form of stable filler material \(^{[275]}\). It is possible to prevent SM collapse by suturing the membrane internally to the sinus wall \(^{[49]}\), however the technique would appear to be proof of principle, rather than recommended as a routine procedure.

The review by Tong et al. (1998) \(^{[2]}\) found that the use of autogenous bone was associated with good survival of implants in augmented sinuses. This technique has been identified as the ‘gold
standard” for sinus grafting \cite{276} against which other techniques must be measured. The search for materials which could reduce the need for autogenous bone harvesting and associated morbidity, encouraged investigators to study new materials, using autogenous bone as the control \cite{241, 249, 277}. These have been extensively reviewed by Aghaloo et al. (2007). The author conducted a meta-analysis of published results for over 5000 implants in augmented sinuses, and reported that implants placed into sinus sites grafted with either autogenous bone or xenograft were associated with survival rates of 92% and 95% respectively, over follow-up periods ranging from 12 to 102 months. This review called for more research, citing methodological issues with many studies, but it seems reasonable to conclude that the best candidates for replacing autogenous bone are the xenograft materials.

1.10.6. Validity of use of Bio-Oss®

There are many publications concerning the use of BO. A simple OvidSP search of the terms “Bio Oss” and “Bio-Oss” yielded 560 results. The material has been described as the ‘gold standard’ for bone replacement \cite{12}, just as autogenous bone was described previously. Many of the studies located refer to comparison of BO and other grafting materials, as demonstrated above, and the authors frequently cite the widespread use of BO clinically \cite{189, 245, 278, 279}.

1.10.7. Summary

The use of BO for sinus augmentation is widespread. Survival rates of implants placed into augmented sinuses are reportedly high. However, little is known about the healing of BO-grafted maxillary sinuses in humans or in animal models. Resorption of xenografts used in sinus augmentation is a controversial subject. Some authors consider it desirable, while others take the opposite view. Despite strong evidence that BO is at best minimally resorbed in the human sinus, studies continue to report observation of resorption with little supporting data.

There is an ongoing need for development of models which accurately represent healing of human sinus grafts, in order to optimize current techniques for implant placement, and also assist in the development of new materials with different biological properties \cite{18}.
Chapter 2 Methods and Materials

This section will describe the materials and methods used to compare the healing of grafted sinus sites in the sheep maxillary sinus model.

Ethical approval for this study was obtained from the Otago Animal Ethics Committee as an amendment to a previously granted approval (AEC 50-80).

2.1. Scanning electron microscopy (SEM)

The surfaces of samples of BO and MB were examined using SEM at magnifications of 90x, 500x and 30,000x. The examination was limited to the unbroken surfaces of particles retaining clear morphological features of trabecular bone (Figure 15).

Specimens were prepared by mounting on 10mm aluminium stubs using double sided carbon tape. Mounted specimens were coated with a 10nm layer of gold / palladium using a Emitech K575X peltier-cooled, high resolution sputter coater with carbon coater attachment (EM Technologies Ltd, England)

Samples were examined using a JEOL JSM-6700F, field emission scanning electron microscope (JEOL Ltd, Japan), at an accelerating voltage of 3.0kV.

2.2. Experimental animals

Eight cross bred ewes, were chosen for this study from flocks sourced by the AgResearch, Invermay Breeding Station. Four animals, all two years old, were sourced initially, representing the four and six week healing periods. A further four animals were selected to represent the eight and 12 week healing periods. There was difficulty matching the age of the two groups, and all animals in the second group of sheep were 18 months old. These were the only animals available within the period of the project.
Once selected, the animals were transferred to the AgResearch, Invermay Breeding Station, where their health and suitability for the study was confirmed. All animals were treated to control external and internal parasites, administered a sub-cutaneous polyvalent Clostridial vaccine and had their hooves trimmed \(^{[229]}\). Animals were individually tagged, and maintained on pasture in a secure site until required for surgery.

Sheep were transported to the Hercus Building, University of Otago, by the Supply Officer of the Hercus Taieri Resource Unit, as required for the surgical timeline. All sheep were delivered between 48 and 72 hours prior to surgery, and starved for 24 hours before administration of the general anaesthetic.
2.3. Surgical materials equipment and techniques

2.3.1. Bio-Oss® (BO), (Geistlich, Switzerland)
Standard sterile, commercially packaged BO (Figure 4), (0.25 – 1mm particle size) was used in BO surgery sites. A new vial was opened for each animal.

![Figure 4 Bio-Oss®, in supplied sterile glass vial](image)

Particle size range 0.25 – 1mm

2.3.2. Moa-Bone® (MB) (Molteno Opthalmic, NZ)
Moa Bone® was supplied in the form of a sterile M-Sphere® in its commercial packaging. The sphere was crushed over a stacked series of sieves, (Endecotts, England), under a laminar flow hood, to achieve a similar size profile to that of the BO particles, as stated in the product details. The sieves had a mesh aperture size of 1.68mm, 1.18mm and 0.25mm. Material that collected on the 0.25mm sieve was considered to in the size range 0.25 to 1mm. The resultant particles were collected and transferred back to the standard M-Sphere® packaging and sterilized in a steam autoclave (Mercer 7, Mercer Medical, New Zealand) for a standard cycle of four minutes at 134°C.

The MB particles were used in the same surgical manner as BO. A separate package of the processed material was opened for each animal.
2.3.3. Bio-Gide® (Geistlich, Switzerland)

Standard sterile, commercially packaged Bio-Gide® (12.5mm x 25mm) was used in all surgical sites. A new package was opened for each animal. The membrane was cut into two equal pieces (Figure 5) that were placed, one on top of the other in a bi-laminar style, over the osteotomy defect using the orientation indicated by the manufacturer (Figure 10).

![Bio-Gide® membrane](image)

**Figure 5 Bio-Gide® membrane**

Divided into two equal halves prior to surgical placement.
2.3.4. Piezotome™
A surgical Piezotome™ (Satalec, Acteon, France), was used to create the surgical window in the antral wall of the sheep sinus. The piezotome was used in conjunction with the supplied SL2 diamond coated ball, bone cutting tip (Figure 7b), and sterile saline irrigation, via the supplied internal pumping apparatus. The initial elevation of the Schneiderian membrane was made using the SL3 sinus elevation tip.

2.3.5. Hand instruments
Further development of the SM elevation was made using a series of specialist hand tools, (Sinus Kit, Osstem, Korea).

2.4. Surgical protocols

All surgery was performed in the facilities of the Animal Research Centre, Hercus Building, Otago University Medical School. Standard sterile operating techniques were adopted throughout the study.

2.4.1. Anaesthetic management
A subcutaneous dose of antibiotics (Strepsin 5ml) was administered one hour pre-operatively. Induction of general anaesthesia was obtained with thiopentone, administered via the cephalic vein (20mg /kg until effect). Sheep were arranged on a mobile, adjustable table with a sandbag for head support. The animals were then intubated by a veterinarian and anaesthesia maintained by a mixture of halothane (1-2%) and nitrous oxide/oxygen (1:2). The rumen was compressed with a stomach tube and the contents allowed to drain into a bucket on the floor. Animals were lifted and rotated on the platform to allow access to the alternate surgical site, during surgery.

2.4.2. Surgical pain control
The site was infiltrated with local anaesthetic 2.2ml of Mepivicaine HCl (Xylocaine® 1:20,000 plus adrenaline), to assist with operative haemorrhage control, and a post-operative dose of long acting anaesthetic Bupivicaine HCL 5 mcg/ml (Marcaine®) was administered when wound closure was complete.
2.4.3. Surgical procedure

The surgical procedure was similar to the one performed and described by Haas and co-workers [53, 89, 92].

![Surgical access site](image)

**Figure 6 Diagram of a dry sheep skull, identifying location of surgical site**

The side of the face was close shaved to remove any excess hair. The facial bony antral wall was exposed via a 6-cm-long paramedian sagittal skin incision. The initial incision through the overlying skin was made with an electrosurgical unit (NeoMed 3000A ESU, Solid State Electrosurgery Unit, USA) in ‘cut’ mode. The exposed masseter muscle was then separated by blunt dissection to expose the underlying bony wall. Any bleeding arterioles were cauterised or clamped and good haemorrhage control was maintained throughout all surgeries. One third of the masseter muscle was detached at the site of insertion, and a circular bony window, approximately 1 cm in diameter (Figure 7a), created rostrally with a Satalec Piezotome™ (Figure 7b). Sterile physiologic saline at room temperature was used as a cooling agent during use of the Piezotome™ device. The resultant bone plate was gently detached from the SM, removed and discarded.
Figure 7 Surgical bony window access to the maxillary sinus

(a) Access window of approximately 1cm diameter

(b) Surgical sinus window showing mobility and inward displacement of bone on sinus membrane using Piezotome™ SL2 tip.

The antral membrane was elevated from the buccal bony wall and displaced dorso-cranially with ‘variably bent’ blunt sinus dissectors (Osstem, Korea). A graft of either MB or BO, mixed with approximately one ml of blood from the surgical field (Figures 8a and 8b), was then placed into the resulting pouch, which was developed caudal to the osteotomy site, using a 2cc syringe with the end removed (Figure 9a). Care was taken to avoid damage to the sinus membrane. No perforations of the sinus membrane were observed during any of the surgical interventions.
Figure 8 The test materials immediately prior to surgical placement
(a) Dappen dish with blood and Moa-Bone® graft material
(b) Dappen dish with blood and Bio-Oss® graft material

Figure 9 Application and placement of grafting material
(a) Modified 2cc syringe filled with MB and blood
(b) Grafted maxillary sinus site prior to membrane placement
The site was then covered with a double layer of Bio-Gide®, (Geistlich Switzerland) according to manufacturer’s instructions, and the wound closed. The deep muscle layers were sutured with resorbable 1-0 Vicryl™ (Ethicon™, Inc, USA) and externally with 4-0 Vicryl™.

Figure 10 Placement of Bio-Gide® membrane (1st layer)

2.4.4. Post–operative pain control
Antibiotics, steroid and anti-inflammatory drugs were administered according to the protocol detailed in appendix I. No animal was observed to suffer any undue adverse short or long term event following surgery. One animal was brought to the attention of the primary investigator (Michael Smith) by the animal welfare officer (AWO). This animal appeared to have a larger swelling over one intervention site. On close examination this was determined to be due to a slightly higher surgical entry point, which in turn unduly emphasised the post operative swelling. This animal was closely observed over the next two days, and no problem detected. In agreement with the AWO no further follow-up was required.

2.4.5. Euthanasia
Animals were euthanized as per the protocol described in the amended ethics document AEC 50-80. This protocol, described below, was developed by Duncan (1997), to allow high quality tissue fixation and minimal haemorrhage into the tissues of interest⁷⁹.
2.4.6. Perfusion protocol

General anaesthesia was induced in the animals with intra-venous (i.v.) Thiopentone @ 20mg/kg via the cephalic vein. This was maintained with halothane (1-2 %) and nitrous oxide/oxygen (ratio 1:2).

The animal was laid on a trolley-table in a supine position, with the neck slightly overextended. The exposed neck region was close shaved with mechanical trimmers. A trans-cutaneous incision of approximately 10cm was made, followed by blunt dissection of the underlying strap muscles of the neck to the level of the carotid artery. The carotid arteries were cannulated bi-laterally, using 14G x 2” gauge indwelling catheters (Optiva™, Smiths Medical, UK). Each catheter was ligated firmly, while maintaining patency, to prevent dislodging of the cannula.

An anaesthetic overdose was administered, and the sheep removed to the post-mortem room. The cannulated carotid artery was connected to a one litre, sterile saline (0.9%) bag (Baxter Healthcare Pty Ltd, Australia) to which had been added 1.5ml of 5000 iu heparin. The bag was elevated to 1.5m above the animal’s heart to allow perfusion of the microvasculature. The external jugular veins were severed, to allow free drainage of blood to the floor. Following saline/heparin administration, a further two litres of fresh, chilled 10% Neutral Buffered Formalin (NBF) (BioLab Ltd., New Zealand) was introduced via the same route.
2.4.7. Harvesting

Following death and formalin perfusion, all tissue specimens were harvested and resected *en bloc*.

The mandible was disarticulated and removed, to allow ready access to the maxilla and sinus regions. The nose and cartilaginous regions anterior to the region of interest, and the overlying skin, were dissected off using a large scalpel. The region of interest was identified by locating scarring at the site of the healing antral bone. A standard hacksaw was then used to separate the maxilla from the skull, distal to the last standing molar. The block was divided down the mid-line with the saw and the two halves labeled. Finally, the block was trimmed using a custom-made guillotine, supplied in the post-mortem room.

Specimens were immediately placed in a sealed container of 10% (NBF). Specimens were then further trimmed to isolate the graft site and placed in smaller 100ml pots of 10% NBF for a total period of 48 hours. When not being manipulated, all specimens were held in 10% NBF at 4°C on a slowly gyrating table at 60 rpm. After forty eight hours, the specimens were transferred to a solution of 70% ethanol and held at 4°C until required.

Each specimen was radiographed to confirm and identify the graft location (Figure 11). The specimens were laid out on a Kodak X-omatic® cassette, fitted with a Lanex® fine screen intensifier and filled with Kodak T-mat G/RA film. The cassette was positioned beneath a Rotanode™ (Toshiba, Japan) tubehead, attached to a Schonander (Stockholm, Sweden) Skull Unit radiography machine. The film was exposed for 0.08 seconds at 50mA / 50kVp and processed in an automatic processing machine.

Specimens were then cut vertically, through the middle of the grafted zone, using an Accutom 50, precision table-top cut-off machine fitted with a diamond cut-off wheel (M0D13 127 x 0.4 x 12.7mm), (Struers GmbH, Switzerland), with water cooling. Cutting took approximately five minutes, no samples were observed to dehydrate during cutting and each sample was returned to 70% ethanol for storage immediately the section was cut.
Figure 11 Radiograph of specimen to identify location of graft material prior to sectioning

One half was then embedded in clear acrylic resin and the remaining tissue decalcified and embedded in paraffin (Figures 12 and 13).
Figure 12 Diagram of specimen sectioning.

A = plane of paraffin-embedded sections, B = line of division and C = plane of resin-embedded sections

2.5. Resin-embedded tissue

Specimens selected for resin-embedding were processed according to the protocol attached in the appendix II. This protocol is similar to that described by Donath (1982) [258], and is identical to that used by Duncan (2005) [79].

2.5.1. Resin-embedding protocol

The protocol for embedding is summarized below. Mixtures of methyl methacrylate (MMA) (Sigma Aldrich, USA) MMA I, MMA II & MMA III were made according to the protocol (appendix II).

Glass jars, with tight fitting screw lids slightly larger than the specimens, were part filled with MMA III to a depth of approximately 8mm, and allowed to set one week prior to use.
Specimens were immersed in xylene (Sigma Aldrich, USA) for two days, with two changes of xylene during the time period, on a rotating platform in a fume hood.

Specimens were transferred to MMA monomer for washing and then placed in MMA I for two days. Specimens were then transferred to MMA II for two days. Finally specimens were placed on to the pre-set bases, as described above, accompanied by a single piece of paper inscribed with the identification code for each sample. The jars were filled with MMAIII, sealed with the screw top lid, and allowed to set in the dark surrounded by water to dissipate excess heat.

Following final maturing of the clear acrylic resin, the jars were removed from storage, wrapped in a towel, and the glass gently smashed using a ball ended hammer. The block of resin containing the specimen was cut to a square shape, to aid location and orientation of the specimen prior to final sectioning. The centre of the graft was clearly identified. The block was labeled with a permanent marker and attached to a larger acrylic block, using cyanoacrylate resin to allow sectioning, as well as minimizing potential for damage to the cutting blade.

2.5.2. Sectioning of resin-embedded tissue

Sequential 500 µm sections were cut from each resin-embedded specimen using a Struers Accutom precision table-top cut-off machine fitted with a diamond cut-off wheel (MOD 13 127 x 0.4 x 12.7mm), (Struers, GmbH, Switzerland), and press mounted onto an opaque acrylic base plate using a cyanoacrylate glue.

Depending on the original size of the specimen, between three and eight slides were prepared from each section of resin-embedded tissue. The identifying codes for each specimen were written on the base plate with a permanent marker pen, and further engraved on the reverse side of the plate using a high speed diamond dental drill. The code included the sheep number, and block identifier, as well as which section from the block was mounted – counting ‘1’ as the closest to the centre of the sample, where the two halves were separated as described previously.
Figure 13 Sequence of specimen processing
(a) site of surgery, (b) radiograph of specimen, (c) division of specimen, decalcified resin-embedded tissue, (d) paraffin-embedded tissue (H&E stain), (e) resin-embedded tissue (MacNeal’s tetrachrome/ toluidine blue stain)
The coded, mounted 500\(\mu\)m sections were then ground, using a rotating grinding machine (Tegra-Pol, Struers, GmbH, ZNL Schweiz, Germany) to achieve a final specimen thickness of between 80 and 100\(\mu\)m. This thickness was measured using a digital micrometer (Digital Indicator, Mitutoyo, Japan). Bulk thinning of the specimen was managed by grinding with Silicon Carbide Paper (Struers, GmbH, ZNL Schweiz, Germany), grit sizes #320 to #1200, followed by polishing of ground specimens on a Knuth Rotor (Struers, GmbH, ZNL Schweiz, Germany) to remove scratches using finer grades up to #4000.

### 2.5.3. Staining

Slides were surface stained with one part MacNeal’s tetrachrome (methylene blue, azur II and methyl violet) and two parts toluidine blue, following immersion in formic acid and methanol \[280\]. Details of the formulation of these stains and the staining protocol are attached in appendix II.
2.6. Paraffin-embedded tissue

2.6.1. Decalcification

The decalcification protocol is that followed by Baharuddin (2010) [229], and summarised below. The benefits of this protocol for immunohistochemical investigation of formalin fixed tissue have been demonstrated previously [281]. The authors compared a number of fixation times and decalcification protocols with a large array of antibodies, and found that the effect of prolonged formalin fixation was not significant with appropriate use of antigen unmasking techniques such as heat retrieval. However the choice of decalcification method particularly impacted on the detection of nuclear antigens.

Samples were removed from alcohol pots and placed in 100ml pots of 10% Ethylenediamine tetraacetic acid (EDTA) and returned to storage at 4°C on a gyrating table. The EDTA solution was replaced every three to four days, and the tissue tested for signs of decalcification with pressure from a blunt probe.

Determination of decalcification was performed by oxalate testing [282], as follows (for protocol see appendix III). A 5ml sample of EDTA was taken from the container holding the tissue and treated with concentrated hydrochloric acid (HCl) (Merck, Germany) until the test solution reached pH 3.2 - 3.6. This was mixed with 5ml of 3% di-ammonium oxalate monohydrate extra pure (Merck Germany), and the resultant solution observed for thirty minutes. Presence of cloudiness or particulate in the test solution was noted as evidence of incomplete decalcification. When no precipitate was observed the sample was considered decalcified and transferred to phosphate buffered saline (PBS), (Gibco™ Invitrogen Corporation, New Zealand) for 24 hours at 4°C on a gyrating table.

Specimens were transferred to the pathology laboratory, School of Dentistry, University of Otago, for embedding in paraffin blocks. The decalcified samples were trimmed with a hand held razor until a suitable size for paraffin-embedding.

Samples were processed using the ‘long cycle’ of the Shandon Citadel 1000 processor (Thermo Fisher Scientific, USA). This automatic processor takes samples through a graded series of ethanol solutions, xylene and finally 56°C melting point Paraplast wax. Orientation of specimens was confirmed, and the cut surface where the graft site was previously sectioned identified as
stated previously. The cut surface was placed face down in each. The processed specimens were then embedded molten (56°C) paraffin. The resultant paraffin blocks were then stored in a sealed container at 4°C until required.
2.6.2. Sectioning and slide preparation

Each block was oriented so that the centre of the graft site was exposed and the blocks trimmed so that sections with complete tissue surfaces were obtained. 12 sections of four µm thickness were then taken in series through the grafted site, using a microtome (Microm HM 340E, Germany) with a S35 disposable feather type microtome blade. A wax ribbon was collected and sections floated in a water bath at 59°C before mounting on 3-aminopropyltriethoxysilane (APES)-coated slides (Lab Scientific, Inc., USA). Slides were then numbered one to 12 for identification, in the order that they were cut. Finally the slides were dried for fifteen to twenty minutes at 45°C and then placed in an incubator (Watson Victor Ltd, England) overnight at 50°C.

Slides were thereafter stored in a slide-box at 4°C until required.

2.6.3. H and E

Slides were stained with haematoxylin and eosin by the MedLab unit, 3rd floor, Dental School, University of Otago (for protocol see appendix III).

2.6.4. TRAP (Tartrate Resistant Acid Phosphatase staining)

TRAP staining was performed using an Acid Phosphatase Leukocyte Kit (387A – 1KT, Sigma Aldrich, USA), designed for cytological detection of acid phosphatase in leukocytes from blood, bone marrow and other tissue. The protocol is attached in appendix III, and outlined below.

Slides were de-waxed in xylene (x2), followed by rehydration in a series of graded ethanols (100%, 100%, 90%, 80%, 70% and 50% concentration), and a final rinse in distilled water before being held in a slide container of distilled water at 37 °C.

TRAP solution was prepared, as per the manufacturers recommendations. Slides were treated with 500µl of TRAP solution and incubated for one hour at 37°C, on a rotating table (20rpm), in the dark. Slides were then thoroughly rinsed in distilled water.

One particular slide stained with such high intensity that interpretation of the histology was compromised. The experiment was repeated using the above protocol, but this specimen was exposed to the TRAP solution for only 10 minutes. Treatment was otherwise identical.

2.6.5. Counterstaining and Coverslip

Counterstaining was performed using haematoxylin (5 seconds).
Slides were cover-slipped using aqueous mountant (Merck, Germany) and glass cover slips applied and sealed with nail polish. Mounted specimens were allowed to dry before excess mounting medium was removed with a scalpel blade and the slide polished with a cloth, prior to examination under high power light microscope.
2.7. Immunohistochemistry

There is paucity of literature in relation to established bone-related antibodies for use in the sheep model. It appears that cross-reactivity of products designed for use in other animal models, including humans, are the only means of obtaining working antibodies for most target proteins. For this reason, antibodies that had a wide spectrum of published success were located using the Biocompare website (www.biocompare.com). These were then further investigated with respect to their research application (e.g. western blot, ELISA, immunohistochemistry) and those antibodies deemed likely to succeed were selected.

The methods in this section report only those antibodies for which successful staining protocols were established. Two antibodies were not pursued. Bone Sialoprotein (ab78278; Abnova, Taiwan) was not found to produce positive staining despite combinations of all the above pre and post treatment protocols. A second antibody, Alkaline Phosphatase (PAB 12279; Abnova, Taiwan) was found to stain positively, but it was not able to be optimized within the time-frame of this project.

2.7.1. Pre / post-treatment(s)

Selected antibodies were trialed using a variety of pre-treatment techniques to expose antigenic binding sites. Six techniques were used; heat, ficin, pepsin, trypsin, proteinase K and no pre-treatment. The concentration of the selected antibody was also varied within the prescribed range on the data sheet supplied, and specimens were further trialed against various agents to remove background staining; tween and non-fat milk powder.

2.7.1.1. Heat pre-treatment

The sections were heated at 80°C for 10 minutes in a bath of 0.01M sodium citrate buffer (pH 6).

2.7.1.2. Trypsin pre-treatment

The sections were treated in a 0.1% Trypsin (Digest-All™Kit, Invitrogen, USA) / 0.1% CaCl₂ (pH 7.8) solution for 10 minutes, at room temperature. Trypsin is a serine protease produced in the pancreas of some animals. Trypsin hydrolyses protein.
2.7.1.3. *Ficin pre-treatment*
Sections were exposed to ready to use Ficin (Digest-All™Kit, Invitrogen, USA) for 5 minutes at RT. Ficin is a cysteine endopeptidase, derived from fig latex.

2.7.1.4. *Pepsin pre-treatment*
Sections were exposed to ready to use Pepsin (Digest-All™Kit, Invitrogen, USA) for 5 minutes at room temperature. Pepsin is a proteolytic, gastric enzyme.

2.7.1.5. *Proteinase K pre-treatment*
Sections were exposed for five minutes to pre-formulated Proteinase K (Dako, Denmark). This is a commercial product designed for proteolytic digestion of formalin-fixed, paraffin-embedded sections. Proteinase K is a broad spectrum serine protease derived from extracts of a particular species of fungus.

2.7.1.6. *Post –treatment with tween and non-fat milk powder*
Sections were washed in a bath of 0.1% Tween ‘80’ (Serva Electrophoresis GmbH, Germany), 1% non-fat milk powder (Pams, New Zealand) or combination of both, for three washes totaling 45 minutes.
2.7.2. RUNX 2 immunohistochemistry

RUNX 2 staining was developed using a rabbit polyclonal, antibody (NBP1-01004, Novus Bio, USA). The polyclonal antibody was raised against a synthetic peptide molecule, corresponding to the region of Aspartate 198 in human RUNX2.

Several concentrations of antibody were trialed to optimize cell staining while minimizing background stain. The final concentration of 10µg/ml (1:100 dilution) was determined optimal via visual inspection of selected trial specimens.

Briefly, specimens were de-waxed in xylene, before rehydrating through graded alcohols. Rehydrated specimens were held in PBS and then subjected to heat pre-treatment (as described above) for ten minutes. Sections were then blocked for non-specific staining with 20% goat serum 1% BSA (Fraction V, Sigma Aldrich, USA) / PBS (Gibco™, Invitrogen Corporation, NZ). The primary antibody was pipetted in 100µl aliquots at 10µg/ml and specimens were left to incubate at 4°C overnight.

2.7.3. PCNA immunohistochemistry

PCNA staining was developed using a PCNA, ‘mouse α rat’ monoclonal, antibody (Clone PC-10, M 0879, Dako, Denmark). Several concentrations of antibody were trialed to optimize cell staining while minimizing background stain. The final concentration of 5.25µg/ml (1:100 dilution) was determined as optimal via visual inspection of selected sample sections.

Specimens were de-waxed in xylene, before rehydrating through graded alcohols. Rehydrated specimens were held in PBS and then subjected to heat pre-treatment (as described above) for ten minutes. Sections were then blocked for non-specific staining with 20% rabbit serum in 1% BSA (Fraction V, Sigma Aldrich, USA) / PBS (Gibco™, Invitrogen Corporation, NZ). The primary antibody was pipetted in 100µl aliquots at 5.25µg/ml and specimens were left to incubate at 4°C overnight.

For detailed final protocols for RUNX2 and PCNA see appendix III.

2.7.4. Control tissues

All sections were also stained using appropriate IgG as a negative control, to confirm validity of positive staining results. Negative control protocols are attached in appendix III as part of the relevant immunohistochemical protocol.
Control IgG for each antibody differed according to the species that the original antibody was raised in, and the same concentration of primary antibody and control antibody was used.

Control IgG for RUNX2 experiments was rabbit IgG (sc2027, Santa Cruz Biotechnology, Santa Cruz, USA), at 10µg/ml.

Control IgG for PCNA experiments was Mouse IgG (sc 2025, Santa Cruz Biotechnology, Santa Cruz, USA) at 5.25µg/ml.

2.7.5. Secondary antibodies and development of HRP

Following overnight incubation at 4°C, sections were washed three times with 1%Tween /1% Non-fat milk powder/ PBS, to reduce background staining, prior to exposure to secondary antibodies. The secondary antibody was added at 2µg/ml in 100µl aliquots and left for one hour at room temperature, before thorough washing in PBS.

The secondary antibody for RUNX2 experiments was ‘Goat anti-Rabbit’ H&L- F(ab)2 Fragment, Biotin (ab6012, Abcam, UK).

The secondary antibody for PCNA experiments was Rabbit Polyclonal to Mouse H&L- F(ab)2 Fragment, Biotin (ab5761, Abcam, UK).

Endogenous peroxidase was quenched using 0.3% hydrogen peroxide in methanol for ten minutes, followed by treatment with Strep HRP (streptococcal horse radish peroxidase, Vectastan Elite ABC, Vector Laboratories, Inc, USA). Finally, development was performed using 3, 3’ diaminobenzidine (DAB), (D3939, Sigma Aldrich, USA) and washing in distilled water.

Counterstain and coverslip

Counterstaining was performed using haematoxylin (five seconds), followed by dehydration in graded alcohols and de-waxing in xylene.

Following counterstaining, slides were cover-slipped using Entellan® (Merck, Germany). Mounted specimens were allowed to dry before excess mounting medium was removed with a scalpel blade and the slide polished with a cloth, prior to examination under high power light microscope.
2.8. Imaging of histological sections

2.8.1. Flat bed scanning
High resolution (3200dpi) digital images, at a ratio of 1:1, of both paraffin and resin-embedded sections were made using a flatbed scanner (Epson Perfection, 2480 Photo, Epson Ltd, New Zealand).

2.8.2. Microscopy
Light microscopy (Olympus binocular, variable magnification settings) was used to examine all sections. Magnified images were digitized using a JVC TK 1281 colour video camera, SPOT® digital imaging software and an Apple Macintosh integrated into the microscope set-up. Resultant images were further processed on a Hewlett Packard HDX Premium Personal Computer machine using Adobe® Photoshop® CS5 software.

Two particular regions of interest were identified (Figure 14). These regions were chosen for two reasons.

1. The ability to compare histological data from human studies with our results. Typically these studies report histology from trephined biopsies taken during or following implant placement. As such, the most apical region of the grafted site is not sampled, to avoid perforation of the sinus membrane.

2. The regions chosen represent the portion of the graft most likely to be utilized for implant placement, and are therefore the most relevant to clinical implant dentistry.
The two regions of interest were defined as follows:

1. Graft material directly adjacent to the pre-existing lamellar bone of the sinus antral wall. This region is referred to as Near To pre-existing Bone (NTB) in this thesis.

2. Graft material distant from the pre-existing lamellar bone of the sinus antral wall. This region is referred to as Distant from pre-existing Bone (DFB) in this thesis.

Figure 14 Decalcified paraffin-embedded section showing the two regions of interest

DFB = Distant from pre-existing bone, NTB = Near to pre-existing bone
H&E stain
Where staining was used to identify cells or proteins of particular interest, frequency of staining was characterized as detailed in Table 5:

**Table 5 Slide staining scoring system**

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No stained cells detected in the ROI</td>
</tr>
<tr>
<td>1</td>
<td>Occasional stained cells in the ROI</td>
</tr>
<tr>
<td>2</td>
<td>Many stained cells in the ROI</td>
</tr>
<tr>
<td>3</td>
<td>Most cells stained in the ROI</td>
</tr>
</tbody>
</table>

ROI = region of interest

Under this system, occasional cell staining (score ‘1’) was where positively stained cells in the specimen were located, but these were few in number and sparsely distributed. In contrast, where all, or nearly all, of the cells stained positively in the region of interest a score of ‘3’ was assigned. A score of ‘2’ was assigned where the number of stained cells was greater than category ‘1’ but less than category ‘3’. A score of ‘0’ was assigned where no positively stained cells were located in the ROI.
Chapter 3 Results

3.1. Handling properties

The handling properties of BO and MB were considerably different when removed from their sterile packaging and manipulated in the surgical environment.

The BO material was easily handled. The particles were dispensed from the vial into the dappen dish by tipping. The BO collected at the base of the dish, and when blood from the surgical site was added it was absorbed readily and evenly into the particles. The resulting mixture was simple to manipulate into the modified applicator / syringe.

MB particles, by contrast, were affected significantly by static electricity. The smaller particles were difficult to contain, and spilled around the dappen dish. Some MB particles collected on the sides of the container and were difficult to re-locate to the base with the bulk of the granules. When the blood from the surgical field was added, it did not appear to be absorbed easily, and the two did not mix readily. When introduced to the applicator syringe, the MB particles were hydrophobic with low wettability, in comparison to the BO mixture which had a uniform appearance.

The BO mixture was simple to dispense and prepare for surgical use. The electrostatic properties of MB made it more difficult to manipulate.

3.2. Post-operative recovery

One animal was observed by the animal welfare officer as having a swelling over a single surgical site twenty four hours after surgery. This animal was examined and the surgical entry site determined to be slightly higher than other animals, thus lying over a bony prominence. The animal was further examined at 36 and 48 hours, and no adverse reaction noted. Otherwise, all animals recovered uneventfully following surgery.
3.3. Site harvesting

The grafted region was recovered from all 16 sites. No graft material, in either region of interest, was located in three of the harvested specimens. These specimens, one from four weeks and two from separate eight week animals, were included in all of the staining protocols discussed below, but were not included in any of the reporting.
3.4. SEM examination

The SEM images of the two materials showed striking similarities and differences depending on the level of magnification.

At 90x magnification both samples retained the morphology of the original trabecular bone (Figure 15). The MB material was difficult to handle and the images showed multiple fragments of material as well as the larger pieces. Even though the MB had been graded for size, it appeared that some of the particles had subsequently broken down into smaller pieces.

Figure 15 SEM images of graft materials at low magnification
Bio-Oss\textsuperscript{®} (left) and Moa-Bone\textsuperscript{®} (right). Both graft materials retain the trabecular structure of cancellous bone.
90x magnification
At higher magnification (500x; Figure 16), the BO particles appeared to be evenly dotted by a regular globular deposit. There were some globular type deposits on the MB particles, but these were more sporadic and sparsely distributed. The MB specimens were noticeably covered in what appeared to be tiny holes, indicating a surface porosity greater than the BO particles.

Figure 16 SEM images of graft materials at medium magnification
Bio-Oss® (left) and Moa Bone® (right). The Moa Bone® surface appears covered in tiny holes compared to Bio-Oss®.
500x magnification
At the highest magnification used (30,000x), the surfaces of the materials appeared completely different. The BO surface appeared to be constructed of clusters of rounded particles, (Figure 17), which seemed to be homogenous and granular. In contrast, the MB surface appeared to retain a semblance of a fibrous structure. The MB surface was covered with similar rounded granules to the BO specimen, but these granules were more sparsely distributed.

**Figure 17 SEM images of graft materials at high magnification**

Bio-Oss® (left) and Moa-Bone® (right). The MB surface appears to retain distinctive structural features of bone when compared to BO.

30,000x magnification

**Summary**

The surface of large MB and BO particles were very different, with the MB material appearing significantly more porous than BO material.
3.5. Radiographic examination of specimens

Bio-Oss®

Moa-Bone®

Figure 18 Radiographic comparison of harvested specimens

Sample specimens of Bio-Oss® (L) and Moa-Bone® (R) grafted sites at the test time points. (a) four weeks, (b) six weeks, (c) eight weeks and (d) 12 weeks.

White arrow = osteotomy, blue arrow = graft material, Scale Bar = 5mm
Note the osteotomy site was not discernible, radiographically, after 12 weeks of healing in BO grafted sites.

The test materials were both radio-opaque, appearing as a white speckled region in the radiograph. The amount of graft material varied between specimens, which may account for differences in the amount of speckling. However it was also notable that MB sites became more radiolucent with time, in contrast to BO sites which became more radio-opaque.

The site of the osteotomy, where bone was removed from the sinus wall, was radiolucent, appearing as a dark circle, adjacent to the graft material. The site of the osteotomy was visible in all specimens, at all time points except 165 L and 166L (Figure 18, BO). These were the sites grafted with Bio-Oss®, 12 weeks post-operatively.

The lack of a radiolucent circle in the region of the osteotomy indicates that the surgical access site may have been filled with bone.
3.6. Examination of resin-embedded sections

Resin-embedded tissue was available for all time points; however no graft material was observed in the regions of interest for BO grafted sites at eight weeks. Serial sections were examined and the section containing the most graft material was described in order to be representative of the main body of the graft.

The staining of the graft material and new bone was readily distinguished, although some sections stained more intensely. Occasional yellow background staining was observed, especially close to the Schneiderian membrane.

Results are summarised in Tables 6 and 7 followed by descriptions and images from each time point.
Table 6 Summary of histological observations for resin-embedded sections where Bio-Oss® was located in the two regions of interest

<table>
<thead>
<tr>
<th>Sheep ID number</th>
<th>Healing time point</th>
<th>Tissue directly surrounding graft material near to pre-existing bone</th>
<th>Tissue directly surrounding graft material distant from pre-existing bone</th>
<th>Resorption lacunae</th>
</tr>
</thead>
<tbody>
<tr>
<td>158 L</td>
<td>4 weeks</td>
<td>WB / CT</td>
<td>CT / WB</td>
<td>N</td>
</tr>
<tr>
<td>151 L</td>
<td>6 weeks</td>
<td>WB</td>
<td>CT / WB</td>
<td>N</td>
</tr>
<tr>
<td>153 L</td>
<td>6 weeks</td>
<td>LB / WB / CT</td>
<td>WB / CT</td>
<td>N</td>
</tr>
<tr>
<td>165 R</td>
<td>12 weeks</td>
<td>LB</td>
<td>WB / LB</td>
<td>N</td>
</tr>
<tr>
<td>166 R</td>
<td>12 weeks</td>
<td>LB</td>
<td>LB</td>
<td>N</td>
</tr>
</tbody>
</table>

Note that no Bio-Oss® particles were located in either region of interest for three specimens, 157 L, 163R and 164 R.
Tissues associated with graft material are listed with the predominant tissue first and the least observed tissue last
WB = woven bone, LB = lamellar bone, CT = connective tissue, Y = yes, N = no
Table 7 Summary of histological observations for resin-embedded sections where Moa-Bone® was located in the two regions of interest

<table>
<thead>
<tr>
<th>Sheep ID number</th>
<th>Healing time point</th>
<th>Tissue directly surrounding graft material near to pre-existing bone</th>
<th>Tissue directly surrounding graft material distant from pre-existing bone</th>
<th>Resorption lacunae</th>
</tr>
</thead>
<tbody>
<tr>
<td>157 R</td>
<td>4 weeks</td>
<td>WB / CT</td>
<td>CT</td>
<td>Y</td>
</tr>
<tr>
<td>158 R</td>
<td>4 weeks</td>
<td>CT / WB</td>
<td>CT / WB</td>
<td>Y</td>
</tr>
<tr>
<td>151 R</td>
<td>6 weeks</td>
<td>WB / LB</td>
<td>CT / WB</td>
<td>Y</td>
</tr>
<tr>
<td>153 R</td>
<td>6 weeks</td>
<td>WB / LB / CT</td>
<td>CT / WB</td>
<td>Y</td>
</tr>
<tr>
<td>163 L</td>
<td>8 weeks</td>
<td>WB / LB</td>
<td>CT / WB</td>
<td>Y</td>
</tr>
<tr>
<td>164 L</td>
<td>8 weeks</td>
<td>WB / LB</td>
<td>CT / WB</td>
<td>Y</td>
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<tr>
<td>165 L</td>
<td>12 weeks</td>
<td>LB</td>
<td>WB / CT</td>
<td>Y</td>
</tr>
<tr>
<td>166 L</td>
<td>12 weeks</td>
<td>LB</td>
<td>WB / CT</td>
<td>Y</td>
</tr>
</tbody>
</table>

Tissues associated with graft material are listed with the predominant tissue first and the least observed tissue last.

WB = woven bone, LB = lamellar bone, CT = connective tissue, Y = yes, N = no
3.6.1. Description of histology for resin-embedded sections

3.6.1.1. Four weeks

Bio-Oss®

BO particles near to pre-existing bone (NTB) were largely engulfed in woven bone (WB), whereas particles distant from pre-existing bone (DFB) were partially surrounded by either WB or CT. The surfaces of the BO particles showed no signs of resorption (Figures 19a and 20a).

Moa-Bone®

Adjacent to the pre-existing sinus wall, small but regular quantities of WB were in close proximity to the particle surfaces. In the second region of interest (DFB) the MB particles were largely surrounded by CT. Only occasional MB particles were associated with WB. Resorption lacunae, i.e. sharply delineated semi-circular pits on the particle surfaces, were regularly detected (Figures 19b and 20b).

3.6.1.2. Six weeks

Bio-Oss®

Particles of BO were almost completely embedded in bone in both regions of interest. Near to pre-existing bone (NTB), the bone was a mixture of lamellar and woven bone, whereas further away from the pre-existing sinus wall, in the region DFB, the particles were associated with only woven bone. No signs of resorption pits on the surfaces of the BO particles were detected in either region (Figures 21a and 22a).

Moa-Bone®

NTB, particles were surrounded by a mixture of WB and bone showing signs of increasing structure and some lamellae with some CT. Distant from the sinus wall MB particles were largely surrounded by connective tissue, with occasional small areas of woven bone close to the particles. Resorption pits on the surfaces of MB particles were common in the region DFB, and to a lesser extent NTB (Figures 21b and 22b).
3.6.1.3. Eight weeks

Bio-Oss®

No BO particles were located in the resin-embedded sections for either region of interest at this time point.

Moa-Bone®

Adjacent to the pre-existing sinus wall, particles were encased in WB, LB or a mixture of both. In the second region of interest, (DFB) tissue surrounding the particle was more variable, either CT or WB. Distinctive resorption lacunae were regularly observed on the MB particle surfaces (Figures 23 and 24).

3.6.1.4. 12 weeks

Bio-Oss®

BO particles in both regions were surrounded by LB and some small regions of WB. Areas of CT lay between the LB but not in contact with the BO particles. No obvious resorption pits were located in either NTB or DFB (Figures 25a and 26a).

Moa-Bone®

Close to the pre-existing bone, MB particles were surrounded by lamellar bone (LB). The particles appeared much smaller in size and fewer in number than at previous time points. Distant from the pre-existing bone, the particles were surrounded by a mixture of WB, LB and CT. Some surfaces appeared affected by resorption pits in DFB although these were difficult to identify as they were smaller and shallower than had been observed at earlier time points (Figures 25b and 26b).
Figure 19 Resin-embedded tissue, near to pre-existing bone, at four weeks
(a) Bio-Oss® (BO) particles near to pre-existing bone (LB), in a mixed environment of connective tissue (CT) and small amounts of lamellar and woven bone (WB).
(b) Moa-Bone® (MB) particles associated with CT with bridges of woven bone (WB) extending out from the underlying pre-existing lamellar bone (LB) associated with the sinus wall. A resorption pit is indicated by the red arrow.
Stained with MacNeal’s tetrachrome/toluidine blue. Scale Bar = 100µm

Figure 20 Resin-embedded tissue, distant from pre-existing bone, at four weeks
(a) Small amounts of woven bone (WB) extending around the Bio-Oss® (BO) particles which are otherwise surrounded by connective tissue (CT).
(b) Moa-Bone® (MB) particles completely surrounded by CT. Large numbers of deep resorptive pits (red arrows) covered the surfaces of the MB.
Stained with MacNeal’s tetrachrome/toluidine blue. Scale Bar = 100µm
Figure 21 Resin-embedded tissue, near to pre-existing bone, at six weeks
(a) Bio-Oss® (BO) particles near to pre-existing bone surrounded by woven bone (WB), lamellar bone (LB) with large areas of connective tissue (CT) surrounding the LB.
(b) Moa-Bone® (MB) particles largely surrounded by woven bone (WB). The MB particle surfaces in contact with connective tissue (CT) were covered with multiple shallow resorption lacunae (red arrows).

Figure 22 Resin-embedded tissue, distant from pre-existing bone, at six weeks
(a) Bio-Oss® (BO) particles distant from pre-existing bone surrounded by woven bone (WB) and lamellar bone (LB) with large areas of connective tissue (CT) surrounding the LB.
(b) Moa-Bone® (MB) nearly completely surrounded by connective tissue (CT). Small amounts of woven bone (WB) were attached to occasional MB particles. MB surfaces were regularly pitted by resorption lacunae (red arrows).

Figures 21 and 22 Stained with MacNeal’s tetrachrome/toluidine blue. Scale Bars = 100µm
Figure 23 Resin-embedded tissue, near to pre-existing bone, at eight weeks

Moa-Bone® (MB) particles largely surrounded by lamellar bone (LB), some woven bone (WB) and connective tissue (CT) evident.

Stained with MacNeal’s tetrachrome/toluidine blue. Scale Bar = 100µm

Figure 24 Resin-embedded tissue, distant from pre-existing bone, at eight weeks

Moa-Bone® nearly completely surrounded by CT. Small amounts of woven bone (WB) were attached to some MB particles. MB surfaces were regularly pitted by resorption lacunae (red arrows).

Stained with MacNeal’s tetrachrome/toluidine blue. Scale Bar = 100µm
Figure 25 Resin-embedded tissue, near to pre-existing bone, at 12 weeks
(a) Bio-Oss® (BO) particles adjacent to pre-existing bone were completely surrounded by lamellar bone (LB) with pockets of connective tissue (CT).
(b) Moa-Bone® (MB) particles were completely surrounded by lamellar bone (LB), with areas of connective tissue (CT). The particles of MB in this region were all very small. Stained with MacNeal’s tetrachrome/toluidine blue. Scale Bar = 100µm

Figure 26 Resin-embedded tissue, distant from pre-existing bone, at 12 weeks
(a) Bio-Oss® (BO) particles distant from pre-existing bone were completely surrounded by lamellar bone (LB) with pockets of connective tissue (CT).
(b) Moa-Bone® (MB) particles were small and surrounded by woven bone (WB) and connective tissue (CT).

Stained with MacNeal’s tetrachrome/toluidine blue. Scale Bar = 100µm
3.7. Examination of decalcified paraffin-embedded sections

The effect of processing on decalcified paraffin-embedded tissues was marked. Lifting or folding of tissue and separation of different tissue types, creating artifactual spaces was common. Tissue section damage did not impact significantly on visual assessment, although it did impact on the ability to record images from the regions of interest. The artifactual nature of the spaces was confirmed by comparing the paraffin-embedded specimens with the resin-embedded tissue (Figure 27).

![Figure 27 Comparison of resin-embedded and decalcified-paraffin-embedded sections](image)

(a) Resin-embedded tissue and (b) decalcified paraffin-embedded tissue from the same specimen. Red arrows indicate artifactual space around graft material in paraffin-embedded specimens.
(b) MacNeal’s tetrachrome/toluidine blue (a), H&E (b) Scale Bar = 100µm

TRAP staining of MB sections at early time points was particularly intense with the red stained connective tissue commonly observed in regions with dense concentrations of osteoclasts (e.g. Figure 31). One section (158 R) was exposed to the TRAP stain for only ten minutes instead of one hour to reduce the generalization of the red stain and allow location of osteoclasts. The control slides confirmed the positive RUNX2 stain of osteoblasts, osteocytes and osteoclasts, as well as undifferentiated cells in the fibrous connective tissue of the consolidating graft.

PCNA staining was discrete and clearly visible using light microscopy. Results are summarised in Tables 8, 9, 10 and 11 followed by description and images from each time point.
3.7.1. Control Tissues

**Figure 28 Control Sections for RUNX2 Staining**

(a) The regular, cuboidal osteoblasts (black arrows) lining the edges of the newly formed woven bone (WB) stain brown following immunohistochemical staining with RUNX2.

(b) Control IgG stained tissue from same region as (a). No brown stained cells can be identified. Counterstained with haematoxylin, scale bar = 100µm

**Figure 29 Control Sections for PCNA Staining**

(a) Positive, brown stained cells (blue arrows) in connective tissue (CT) surrounding Moa-Bone (MB) graft particles following immunohistochemical staining with PCNA. The positive PCNA staining is exclusive to the nucleus of positively stained cells.

(b) Control IgG stained tissue from same region as (a). No brown stained cells can be identified. Counterstained with haematoxylin, scale bar = 100µm
Table 8 Summary of histological observations for decalcified paraffin-embedded specimens where Bio-Oss® graft material was located near to pre-existing bone

<table>
<thead>
<tr>
<th>Sheep ID Number</th>
<th>Healing Time Point</th>
<th>Tissues Associated With Graft Particles</th>
<th>TRAP Positive Cells</th>
<th>Resorption Lacunae</th>
<th>RUNX2 Positive Cells in connective tissue Surrounding Graft</th>
<th>PCNA Positive Cells in connective tissue Surrounding Graft</th>
</tr>
</thead>
<tbody>
<tr>
<td>158 L</td>
<td>4 weeks</td>
<td>WB</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>151 L</td>
<td>6 weeks</td>
<td>LB / CT</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>153 L</td>
<td>6 weeks</td>
<td>LB/WB/CT</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>165 R</td>
<td>12 weeks</td>
<td>LB/WB/CT</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>166 R</td>
<td>12 weeks</td>
<td>LB</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Note that no BO material was located in this region for three specimens, 157 L, 163 R and 164 R. No specimen containing BO was available for analysis at eight weeks of healing.

Tissues associated with graft material are listed with the predominant tissue first and the least observed tissue last.

- WB = woven bone, LB = lamellar bone, CT = connective tissue
- 0 = no positive cells / lacunae detected, 1 = occasional positive cells / lacunae detected, 2 = many positive cells / lacunae detected, 3 = most cells of the region positively stained or most particles of the region affected by resorption lacunae.
Table 9 Summary of histological observations for decalcified paraffin-embedded specimens where Moa-Bone® material was located near to pre-existing bone

<table>
<thead>
<tr>
<th></th>
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<td>157 R</td>
<td>4 weeks</td>
<td>CT</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>158 R</td>
<td>4 weeks</td>
<td>CT/WB</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>151 R</td>
<td>6 weeks</td>
<td>WB/CT</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>153 R</td>
<td>6 weeks</td>
<td>CT</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>163 L</td>
<td>8 weeks</td>
<td>CT/WB</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>164 L</td>
<td>8 weeks</td>
<td>CT/WB</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>165 L</td>
<td>12 weeks</td>
<td>LB/CT</td>
<td>1</td>
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<td>0</td>
</tr>
<tr>
<td>166 L</td>
<td>12 weeks</td>
<td>LB/WB /CT</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Tissues associated with graft material are listed with the predominant tissue first and the least observed tissue last

WB = woven bone, LB = lamellar bone, CT = connective tissue

0 = no positive cells / lacunae detected, 1 = occasional positive cells / lacunae detected, 2 = many positive cells / lacunae detected, 3 = most cells of the region positively stained or most particles of the region affected by resorption lacunae
3.7.2. Descriptions of healing around graft particles near to pre-existing bone

3.7.2.1. Four Weeks

Bio-Oss®

Tissue on the section available was significantly distorted and folded on the slide. The BO particles were surrounded by woven bone with some areas appearing more structured and lamellar in nature (Figure 30a). A small amount of the particle surfaces were still in contact with connective tissue. Occasional lightly TRAP positive stained multinuclear cells were located on the surface of the particles still exposed to connective tissue, although there was no sign of resorption of the particles beneath these cells (Figure 31a). Osteoblasts on the surface of woven bone stained positively to RUNX2 but the CT was devoid of cells staining positive for RUNX2 or PCNA (Figures 32a and 33a).

Moa-Bone®

The MB particles were surrounded by soft connective tissue with occasional small amounts of woven bone (Figure 30b). The particles were consistently associated with large numbers of multinucleate cells, often in clearly defined resorption pits, which stained intensely with TRAP, as did the extracellular matrix (Figure 31b).

RUNX2 positive stained osteocytes were identified in the woven bone near the MB particles. Osteoblasts on the surface of the woven bone were stained clearly, while the cytoplasm of cells in the surrounding connective tissue was lightly, but regularly, positively stained brown, as were multinucleated cells suggestive of osteoclasts (Figure 32b). Small numbers of PCNA positive cells were identified amongst the cells of the surrounding connective tissue (Figure 33b).
Figure 30 H&E staining at 4 weeks, near to pre-existing bone
(a) Bio-Oss® (BO) particles embedded in a composite environment of woven bone (WB) and lamellar bone (LB). The connective tissue is less cellular than (b).
(b) Moa-Bone® (MB) particles surrounded by a mixture of woven bone (WB) and a densely cellular fibrous connective tissue (CT). The WB overlies the pre-existing lamellar bone (LB) of the sinus wall.
Scale Bar = 50µm

Figure 31 TRAP staining at 4 weeks, near to pre-existing bone
(a) Bio-Oss® (BO) particles surrounded by large amounts of woven bone (WB). Occasional TRAP staining cells (red arrows) located in the connective tissue.
(b) Moa-Bone® (MB) particles associated with multiple, intensely stained TRAP positive cells (red arrows) and resorption lacunae where exposed to the surrounding connective tissue (CT). The CT also stains red, especially adjacent to osteoclasts.
Sections counterstained with haematoxylin. Scale bar = 100µm
**Figure 32 Immunohistochemical labeling with RUNX2 at 4 weeks, near to pre-existing bone**

(a) Brown, RUNX2, stained osteoblasts (black arrows) line the woven bone (WB) of the loose connective tissue (CT) surrounding the Bio-Oss® (BO) particles.

(b) The fibrous connective tissue (CT) surrounding the Moa-Bone® (MB) particles is filled with numerous RUNX2 positive stained cells.

Sections counterstained with haematoxylin. Scale bar = 100µm

**Figure 33 Immunohistochemical labeling with PCNA at 4 weeks, near to pre-existing bone**

(a) No PCNA positive cells were detected in the Bio-Oss® (BO) specimens in this region.

(b) Occasional lightly stained PCNA positive cells (black arrows) in the fibrous connective tissue of the Moa-Bone® (MB) section.

Sections counterstained with haematoxylin. Scale bar = 100µm
3.7.2.2. Six Weeks

**Bio-Oss®**

BO particles were nearly completely surrounded by bone, most of which was lamellar in structure (Figure 34a). No TRAP or PCNA positive cells were located near the particles (Figures 35a and 37a), however the cells lining the bone stained positively for RUNX2 (Figure 36a).

**Moa-Bone®**

MB particles were partially surrounded by small amounts of woven bone, extending out from the lamellar bone beneath, and connective tissue on the outermost surface (Figure 34b). Intensely stained TRAP positive multinuclear cells were regularly located where the particles were still in contact with the connective tissue (Figure 35b). The cells of the connective tissue regularly stained positive to RUNX2 and PCNA (Figures 36b and 37b). Multi-nucleated cells stained positive for RUNX2. In Figure 39a it is possible to compare a RUNX2 positive brown stained cell with the TRAP stained section, indicating that these cells are osteoclasts.
Figure 34 H&E staining at 6 weeks, near to pre-existing bone
(a) Bio-Oss® (BO) particles embedded in a composite environment of woven bone (WB) and lamellar bone (LB). The connective tissue (CT) is less cellular than (b).
(b) Moa-Bone® (MB) particles surrounded by a mixture of woven bone and a densely cellular fibrous connective tissue. Woven bone overlies the pre-existing lamellar bone of the sinus wall.
Scale Bar = 50µm

Figure 35 TRAP staining at 6 weeks, near to pre-existing bone
(a) Bio-Oss® (BO) particles nearly surrounded by bone. Few TRAP stained cells (red arrow) were located in the connective tissue (CT) in this region.
(b) Moa-Bone® (MB) particles regularly associated with heavily stained TRAP positive cells (red arrows) and resorption lacunae where graft material was exposed to the surrounding connective tissue. Where new bone was near to the graft material no osteoclasts were observed.
Counterstained with haematoxylin, Scale Bar = 100µm
Figure 36 Immunohistochemical labeling with RUNX2 at 6 weeks, near to pre-existing bone
(a) Darkly stained RUNX2 osteoblasts (black arrows) line the lamellar bone (LB) which surrounds the Bio-Oss® (BO) particles. No cells in the connective tissue (CT) stain positively for RUNX2. Mature osteocytes in the lamellar bone do not stain brown for RUNX2 (red arrow).
(b) Darkly positive RUNX2 osteoblasts line the lamellar bone surrounding the Moa-Bone® (MB) particles. The cytoplasm of most of the cells of the surrounding connective tissues stain positively light brown (white arrows), to RUNX2.
Counterstained with haematoxylin, Scale Bar = 100µm

Figure 37 Immunohistochemical labeling with PCNA at 6 weeks, near to pre-existing bone
(a) No PCNA positive cells located in the connective tissue (CT) near Bio-Oss® (BO) in the region.
(b) Many PCNA positive cells (black arrows) located in the connective tissue (CT) near the lamellar bone (LB) surrounding the Moa-Bone® (MB) particles.
Counterstained with haematoxylin, Scale Bar = 100µm
3.7.2.3. *Eight Weeks*

**Bio-Oss®**

Neither specimen collected at eight weeks yielded any BO particles in the region of interest.

**Moa-Bone®**

The MB particles were surrounded by a densely cellular fibrous connective tissue (Figure 38a). There was no sign of new bone extending out from the pre-existing mature bone adjacent to the particles. TRAP positive cells were regularly observed in close association with the MB surfaces, usually in depressions or clear resorptive pits (Figure 38b). The cytoplasm of cells in the fibrous connective tissue regularly stained positively for RUNX2, as did the osteocytes of the adjacent lamellar bone (Figure 39a). PCNA staining revealed occasional positive cells in the fibrous connective tissue surrounding the MB material (Figure39b).
Figure 38 H&E and TRAP staining of Moa-Bone® at 8 weeks, near to pre-existing bone

(a) Moa-Bone® particles (MB) in a densely cellular fibrous connective tissue (CT), alongside the pre-existing lamellar bone (LB).

(b) TRAP positive cells (red arrows) regularly dot the MB particles beneath which are clear signs of resorptive pits.

TRAP section (b) counterstained with haematoxylin. Scale Bar = 50µm (a); 100µm (b)

Figure 39 Immunohistochemical labeling of Moa-Bone® at 8 weeks, near to pre-existing bone with (a) RUNX2 and (b) PCNA

(a) RUNX2 positive, brown stained cells (black arrows) in the fibrous connective tissue (CT) surrounding the Moa-Bone® particles (MB). Brown stained multi-nucleated cells in the same location as cells previously identified as osteoclasts (red arrows).

(b) Occasional brown stained, PCNA positive cell (blue arrows) in the connective tissue surrounding the MB from the same area of the specimen as described in (a).

Sections counterstained with haematoxylin. Scale bar = 100µm
3.7.2.4. 12 Weeks

Bio-Oss®

BO material was embedded in lamellar bone with regularly spaced osteocytes. Some regions of irregular woven bone were observed between regions of lamellar bone. Occasional particles of BO were noted to be facing connective tissue (Figure 42a). TRAP positive cells were observed only adjacent to these particles however no clear resorptive pits were noted to be associated with these osteoclasts (Figure 41a). Lightly RUNX2 positive, osteoblasts lined the boundary between the connective tissue and the lamellar bone; and small numbers of positive stained cells were located in the connective tissue matrix. Small numbers of the cells in the connective tissue located around BO particles but otherwise enclosed by bone were identified as staining positive for PCNA.

Moa-Bone®

MB particles were surrounded by bone showing clear lamellae but with irregular osteocyte distribution (Figure 40b). The regions of bone were interspersed with connective tissue, some of which was in contact with small areas of the MB material. The cells lining the lamellar bone surrounding the residual graft particles stained deeply to RUNX2 antibodies, with occasional cells in the surrounding connective tissue matrix staining light brown (Figure 42b). Occasional TRAP positive cells were observed only where particles were in contact with connective tissue, with no obvious resorptive pits associated with these cells. No TRAP positive cells were detected near MB particles surrounded by bone (Figure 41b). PCNA positive staining cells were not observed near the MB particles or the surrounding tissues.

Only small numbers of MB particles were observed. All particles of MB appeared small in comparison to those observed at earlier time points.
Figure 40 H&E staining at 12 weeks, near to pre-existing bone

(a) Bio-Oss® (BO) particles surrounded by lamellar bone (LB) and a loose fibrous connective tissue (CT).
(b) Moa-Bone® (MB) particles surrounded by lamellar bone, similar to (a). There is a greater proportion of connective tissue in (b) than (a).
Scale Bar = 50µm

Figure 41 TRAP staining at 12 weeks, near to pre-existing bone

(a) Some Bio-Oss® (BO) particles were associated with TRAP positive osteoclasts (red arrow). Osteoclasts were only detected where the particle was exposed to the connective tissue. There were no signs of resorptive pits on the surface of the particles.
(b) The few Moa-Bone® particles detected were completely embedded in lamellar bone (LB). No TRAP positive cells were located in the region of interest.
Sections counterstained with haematoxylin. Scale bar = 100µm
Figure 42 Immunohistochemical labeling with RUNX2 at 12 weeks, near to pre-existing bone
(a) Brown stained RUNX2 positive osteoblasts (black arrows) border the lamellar bone (LB) surrounding the Bio-Oss® (BO) particles. Occasional RUNX2 positive stained cells (purple arrows) in the connective tissue (CT) of this section.
(b) Brown stained RUNX2 positive osteoblasts line the lamellar bone (LB) enveloping the Moa-Bone® (MB) particles. The connective tissue (CT) contains few RUNX2 positive cells.
Sections counterstained with haematoxylin. Scale bar = 100µm

Figure 43 Immunohistochemical labeling with PCNA at 12 weeks, near to pre-existing bone
(a) Occasional PCNA positive cells (black arrows) were located in the connective tissue (CT) in the region of Bio-Oss® (BO) particles (a), and Moa-Bone® (MB) particles (b).
Figure 42(a) is the same region depicted as in figure 41(a), with different immunohistochemical staining.
Sections counterstained with haematoxylin. Scale bar = 100µm
Table 10 Summary of histological observations for decalcified paraffin-embedded specimens where Bio-Oss® was located distant from pre-existing bone

<table>
<thead>
<tr>
<th>Sheep ID Number</th>
<th>Healing Time Point</th>
<th>Tissues Associated With Graft Particles</th>
<th>TRAP Positive Cells</th>
<th>Resorption Lacunae</th>
<th>RUNX2 Positive Cells in CT Surrounding Graft</th>
<th>PCNA Positive Cells in CT Surrounding Graft</th>
</tr>
</thead>
<tbody>
<tr>
<td>158 L</td>
<td>4 weeks</td>
<td>WB</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>151 L</td>
<td>6 weeks</td>
<td>LB / CT</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>153 L</td>
<td>6 weeks</td>
<td>LB/WB/CT</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>165 R</td>
<td>12 weeks</td>
<td>LB/WB</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>166 R</td>
<td>12 weeks</td>
<td>LB</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Note that no graft material was located in this region for three specimens, 157 L, 163 R and 164 R. No specimen containing BO was available for analysis at eight weeks of healing.

Tissues associated with graft material are listed with the predominant tissue first and the least observed tissue last.

WB = woven bone, LB = lamellar bone, CT = connective tissue

0 = no positive cells / lacunae detected, 1 = occasional positive cells / lacunae detected, 2 = many positive cells / lacunae detected, 3 = most cells of the region positively stained or most particles of the region affected by resorption lacunae.
Table 11 Summary of histological observations for decalcified paraffin-embedded specimens where Moa-Bone® material was located distant from pre-existing bone

<table>
<thead>
<tr>
<th>Sheep ID Number</th>
<th>Healing Time Point</th>
<th>Tissues Associated With Graft Particles</th>
<th>TRAP Positive Cells</th>
<th>Resorption Lacunae</th>
<th>RUNX2 Positive Cells in CT Surrounding Graft</th>
<th>PCNA Positive Cells in CT Surrounding Graft</th>
</tr>
</thead>
<tbody>
<tr>
<td>157 R</td>
<td>4 weeks</td>
<td>CT</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>158 R</td>
<td>4 weeks</td>
<td>CT/WB</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>151 R</td>
<td>6 weeks</td>
<td>WB/CT</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>153 R</td>
<td>6 weeks</td>
<td>CT</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>163 L</td>
<td>8 weeks</td>
<td>CT/WB</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>164 L</td>
<td>8 weeks</td>
<td>CT/WB</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>165 L</td>
<td>12 weeks</td>
<td>LB/CT</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>166 L</td>
<td>12 weeks</td>
<td>LB/WB/CT</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Tissues associated with graft material are listed with the predominant tissue first and the least observed tissue last.

WB = woven bone, LB = lamellar bone, CT = connective tissue

0 = no positive cells / lacunae detected, 1 = occasional positive cells / lacunae detected, 2 = many positive cells / lacunae detected, 3 = most cells of the region positively stained or most particles of the region affected by resorption lacunae.
3.7.3. Descriptions of healing around graft particles distant from pre-existing bone

3.7.3.1. Four Weeks

**Bio-Oss®**

Most of the BO material was surrounded by small amounts of irregular woven bone with small regions beginning to appear lamellar in structure. Some of the woven bone appeared to form thin extensions between BO particles in an irregular connective tissue matrix (Figure 44a). Small numbers of TRAP positive cells were observed on the surface of some particles. These osteoclasts did not appear to be associated with resorption of the BO although they were closely adapted to the surface of the graft material (Figure 45a). The regions of woven bone connecting the BO particles were lined with cells staining positive for RUNX2 (Figure 46a). Cells staining lightly for RUNX2 were only occasionally located in the surrounding connective tissue and no PCNA positive staining cells were located near the graft material (Figure 47a).

**Moa-Bone®**

The MB particles were largely surrounded by a densely cellular fibrous connective tissue, with small amounts of woven bone near some particles (Figure 44b). Numerous heavily stained TRAP positive cells dotted the surface of the MB in clearly defined resorptive lacunae (Figure 45b). The cells of the fibrous connective tissue surrounding the MB stained positive for RUNX2 in tissue from only one specimen. The cells closest to the areas of new bone stained more deeply for RUNX2 than those further away. Regular numbers of PCNA positive cells were located in the fibrous connective tissue closest to the MB particles, in the same region as the darker staining osteoblasts previously identified with RUNX2 staining.
**Figure 44 H&E staining at 4 weeks, distant from pre-existing bone**

(a) Bio-Oss® (BO) particles embedded in a mixture of woven bone (WB) and loose connective tissue (CT). The connective tissue (CT) is less cellular than (b).
(b) Moa-Bone® (MB) particles surrounded by small amounts of woven bone and a densely cellular fibrous connective tissue.

Sections counterstained with haematoxylin. Scale bar = 100µm

**Figure 45 TRAP staining at 4 weeks, distant from pre-existing bone**

(a) Bio-Oss® (BO) particles in a mixture of woven bone (WB) and fibrous connective tissue (CT). Osteoclasts (red arrows) were located where the connective tissue bounded the BO particles, but no resorptive pits can be seen on the particle surfaces.
(b) Moa-Bone® (MB) particles associated with osteoclasts and deep resorption lacunae where exposed to the surrounding connective tissue (CT).

Sections counterstained with haematoxylin. Scale bar = 100µm
Figure 46 Immunohistochemical labeling with RUNX2 at 4 weeks, distant from pre-existing bone

(a) Dark RUNX2 stained osteoblasts (black arrows) line the lamellar bone (LB) surrounding the Bio-Oss® (BO) particles. No cells in the connective tissue (CT) stain positively for RUNX2.
(b) Numerous lightly stained RUNX2 positive cells (blue arrows) in the connective tissues surrounding the Moa-Bone® (MB). Darker stained osteoblasts (black arrows) line the small amounts of woven bone (WB) adjacent to the MB particles.
Sections counterstained with haematoxylin. Scale bar = 100µm
Figure 47 Immunohistochemical labeling with PCNA at 4 weeks, distant from pre-existing bone

(a) No PCNA positive stained cells in the connective tissue (CT) adjacent to the woven bone (WB) surrounding the Bio-Oss® (BO) particles in the region.
(b) Numerous PCNA positive cells (black arrows) in the connective tissues surrounding the Moa-Bone® (MB), and the small amounts of adjacent woven bone (WB).

Sections counterstained with haematoxylin. Scale bar = 100µm

Figures 46(a) and (b) are the same regions depicted in figures 45(a) and (b), with different immunohistochemical staining.
3.7.3.2. Six Weeks

Bio-Oss®

The BO particles were located in a mixed network of fibrous connective tissue, woven bone and small amounts of lamellar bone (Figure 48a). Where BO particles were exposed to the fibrous connective tissue, occasional TRAP positive cells were located on the surface of the particle, but these lacked signs of resorptive lacunae (Figure 49a). RUNX2 positive stained cells lined the new bone surrounding the BO particles. Lightly stained RUNX2 positive cells were sparsely distributed in the fibrous connective tissue surrounding the BO particles (Figure 50a), but PCNA positive cells were not located in the region of interest (Figure 51a).

Moa-Bone®

The MB particles were located in a predominantly densely cellular fibrous connective tissue, with some woven bone adjacent to some particles (Figure 48b). The MB particle surfaces were covered by multiple TRAP positive cells, which lay in distinctive resorption pits (Figure 49b). RUNX2 positive cells were regularly located in the fibrous connective tissue surrounding MB particles. Cells on the surface of new woven bone stained more intensely than those of the surrounding fibrous connective tissue (Figure 50b). PCNA positive cells were regularly located amongst the cells surrounding the graft particles and new bone (Figure 51b), in the region that had stained positive for RUNX2 (Figure 50b).
**Figure 48 H&E staining at 6 weeks, distant from pre-existing bone**

(a) Bio-Oss® (BO) particles embedded in a mixture of woven bone (WB), lamellar bone (LB) and fibrous connective tissue (CT).

(b) Moa-Bone® (MB) particles surrounded by small amounts of woven bone and a densely cellular, fibrous connective tissue.

Scale Bar = 50µm

**Figure 49 TRAP staining at 6 weeks, distant from pre-existing bone**

(a) Bio-Oss® (BO) particles in a mixture of woven bone (WB), lamellar bone (LB) and fibrous connective tissue (CT). TRAP staining cells (red arrows) were located where the connective tissue bounded the BO particles, but no resorptive pits can be seen on the particle surfaces.

(b) Moa-Bone® (MB) particles associated with TRAP positive cells and deep resorption lacunae where next to the surrounding fibrous connective tissue (CT).

Sections counterstained with haematoxylin. Scale bar = 100µm
**Figure 50 Immunohistochemical labeling with RUNX2 at 6 weeks, distant from pre-existing bone**

(a) Dark RUNX2 stained osteoblasts (black arrows) line the lamellar bone (LB) and woven bone (WB) which surrounds the Bio-Oss® (BO) particles. No cells in the connective tissue (CT) stain positively for RUNX2.

(b) Numerous RUNX2 positive cells in the connective tissues (purple arrows) surrounding the Moa-Bone® (MB), and the small amounts of woven bone (WB) observed. Brown stained multinucleated cell /osteoclast (red arrows).

Sections counterstained with haematoxylin. Scale bar = 100µm
Figure 51 Immunohistochemical labeling with PCNA at 6 weeks, distant from pre-existing bone

(a) Occasional PCNA positive stained cells (black arrows) in the connective tissue (CT) adjacent to the lamellar bone (LB) surrounding the Bio-Oss® (BO) particles in the region.

(b) Numerous PCNA positive cells in the connective tissues (CT) surrounding the Moa-Bone® (MB), and the small amounts of woven bone (WB) observed.

Figure 50(b) is the same region depicted as in figure 49(b), with different immunohistochemical staining.

Sections counterstained with haematoxylin. Scale bar = 100μm
3.7.3.3. *Eight Weeks*

**Bio-Oss**®

Neither specimen collected at eight weeks yielded any BO particles in the region of interest.

**Moa-Bone**®

The MB particles were largely surrounded by a very cellular fibrous connective tissue, with small amounts of woven bone adjacent to some particles (Figure 52a). TRAP positive cells were common in close association with the surfaces of the graft material, which was frequently indented with resorption lacunae beneath the TRAP positive osteoclasts (Figure 52b). RUNX2 positive cells were commonly identified in the fibrous connective tissue. Staining of these cells increased in intensity among cells closer to the new bone forming adjacent to the MB material (Figure 53a). PCNA positive cells were regularly located in the connective tissue surrounding the graft material (Figure 53b). PCNA positive stained cells were more frequent in the region of the more intensely stained RUNX2 positive cells. The proliferative activity of RUNX2 positively stained cells (Figure 53a) can be seen when the same region was stained using PCNA (Figure 53b).
Figure 52 H&E and TRAP staining of Moa-Bone® at 8 weeks, distant from pre-existing bone
(a) Moa-Bone® (MB) particles in densely cellular connective tissue, as shown by H&E staining.
Small amounts of woven bone (WB) detected near the surfaces of some MB particles.
(b) The Moa-Bone® (MB) particles were surrounded by large numbers of osteoclasts (red arrows)
in deep resorption pits wherever the particles were in contact with the fibrous connective tissue (CT).
TRAP section (b) counterstained with haematoxylin, Scale Bar = 50µm (a); 100µm (b)

Figure 53 Immunohistochemical labeling of Moa-Bone® at 8 weeks, distant from pre-existing bone with (a) RUNX2 and (b) PCNA
(a) Numerous RUNX2 positive cells (black arrows) in the connective tissue (CT) surrounding the Moa-Bone® (MB), and along the surface of the woven bone (WB).
(b) Frequent PCNA positive cells (blue arrows) in the connective tissue (CT) surrounding the Moa-Bone® (MB), and adjacent to the surface of the woven bone (WB).
Sections counterstained with haematoxylin. Scale bar = 100µm
3.7.3.4. 12 Weeks

**Bio-Oss®**

The BO particles were mainly embedded in a bony matrix, consisting largely of lamellar bone with small regions of less organized woven bone (Figure 54a). Islands of loose fibrous connective tissue were located throughout the maturing bone. Small numbers of BO particles were surrounded by bone and a more cellular fibrous connective tissue (Figure 56a).

Occasional TRAP positive particles were observed where connective tissue contacted BO particles (Figure 55a). The osteoclasts identified appeared peripheral to the graft material and no sign of resorption was noted. Some BO particles were observed with measurable dimensions of approximately one mm (Figure 55a). This is similar to that of the largest size in the range stated on the product packaging.

The more cellular fibrous connective tissue contained regular numbers of lightly stained RUNX2 positive cells surrounding more heavily stained osteoblasts on the surface of the new bone (Figure 56a). Occasional PCNA positive cells were located in the connective tissue adjacent to the newly formed bone (Figure 57a). No RUNX2 or PCNA positive cells were located in the loose connective tissue similar to that identified in the H & E sections (Figure 54a).

**Moa-Bone®**

The MB particles were located in a mixed environment of lamellar and woven bone surrounded by a richly cellular connective tissue (Figure 54b).

Where the particles were in contact with the fibrous connective tissue, positive TRAP stained cells were regularly observed on the indented surface of the particles, (Figure 55b).

Cells in the fibrous connective tissue and those close to the new bone in contact with the MB stained heavily for RUNX2. Staining for PCNA revealed regular positively stained cells in the region closest to the woven bone. MB particles observed appeared very small, and much smaller than those observed in the BO sections or MB particles observed at week four (Figure 44b).
Figure 54 H&E staining at 12 weeks, distant from pre-existing bone
(a) Bio-Oss® particles surrounded by lamellar bone and islands of loose fibrous connective tissue (CT).
(b) Moa-Bone® (MB) particles in a mixed environment of woven bone (WB), lamellar bone and a densely cellular fibrous connective tissue
(Scale Bar = 50µm)

Figure 55 TRAP staining at 12 weeks, distant from pre-existing bone
(a) Bio-Oss® (BO) particle nearly completely surrounded by lamellar bone (LB). The BO particle was in contact with one of the few osteoclasts (red arrows) identified in the connective tissue of the region.
(b) Multiple osteoclasts in close association with the Moa-Bone® (MB) particles and woven bone (WB) located in the region. Resorption pits beneath osteoclasts appeared broad and shallow. Sections counterstained with haematoxylin. Scale bar = 100µm
Figure 56 Immunohistochemical labeling with RUNX2 at 12 week distant from pre-existing bone

(a) Bio-Oss® (BO) surrounded by lamellar bone (LB). Dark brown stained RUNX2 positive cells (black arrows) were detected in association with the outer surface of the bone. Light brown stained RUNX2 positive cells (purple arrows) were located in the surrounding connective tissue (CT).

(b) Moa-Bone® (MB) particles partially surrounded by woven bone (WB) and connective tissue. RUNX2 positive cells found in and around the woven bone as well as in the connective tissue. The connective tissue was noticeably more cellular than (a)

Sections counterstained with haematoxylin. Scale bar = 100µm
Figure 57 Immunohistochemical labeling with PCNA at 12 weeks, distant from pre-existing bone

(a) Occasional brown stained PCNA positive cells (black arrows) in the connective tissue surrounding the Bio-Oss® (BO) particles.
(b) Frequent, deeply stained PCNA positive cells in the connective tissue (CT) surrounding woven bone and Moa-Bone® (MB) particles in the region.
Sections counterstained with haematoxylin. Scale bar = 100µm
3.8. Summary of analysis for decalcified paraffin-embedded tissue

3.8.1. Bio-Oss®

BO particles in the region NTB were consistently surrounded by new bone at all healing points observed. The new bone surrounding BO particles was increasingly lamellar in structure as the healing time increased. A gradient of consolidation of the graft site, resulting in less mature bone in the region DFB compared to NTB was consistently noted. New bone seemed to be formed only in close proximity to existing bone, which then extended out through the consolidating graft.

Small numbers of TRAP stained osteoclasts were regularly observed on the surface of the BO particles where they were in contact with connective tissue up until six weeks (Figure 49a), and occasionally at 12 weeks (Figure 41a). No resorption lacunae were observed in the region of TRAP stained osteoclasts (Figure 41a). After 12 weeks of healing, no significant resorption of the BO particles was recorded in either of the regions of interest.

RUNX2 stained osteoblasts were regularly observed lining the edges of newly formed bone both NTB and DFB (Figures 36a and 46a). Small numbers of RUNX2 positive cells were observed in the connective tissue surrounding the BO particles in the region DFB at 12 weeks (Figure 56a).

PCNA positive stained cells were occasionally observed in the connective tissue adjacent to bone forming around the BO particles in both regions NTB and DFB.

3.8.2. Moa-Bone®

Healing of sinus grafts using MB was quite different from BO grafts both NTB and DFB, at eight weeks of healing or earlier. At these time points MB was regularly surrounded by woven bone or fibrous connective tissue, only fully surrounded by bone after 12 weeks of healing. The connective tissue surrounding MB particles was very cellular compared to the connective tissue surrounding BO graft particles (Figure 34a and b).

There was a clear temporal gradient in healing, similar to BO, whereby the amount of bone and its maturity associated with the MB particles increased over the course of the experiment. New bone formed earliest near to pre-existing bone of the sinus wall, as with BO grafts. In addition,
new bone formed in the region DFB in the absence of any obvious connection to underlying or other newly formed bone (Figure 44b). In comparison to the healing BO sites the new bone was surrounding the MB particles was less mature at the same healing time point.

TRAP positive osteoclasts were frequently observed in distinct resorption lacunae before new bone formed around the MB particles. At four and six weeks the TRAP staining of the osteoclasts was particularly intense (Figures 31b and 45b). The size of observable MB particles was noticeably smaller after 12 weeks of healing.

The cells of the fibrous connective tissue both NTB and DFB consistently stained positive for RUNX2. There was a gradient of intensity of the RUNX2 staining around MB particles where new bone was forming. Osteoblasts lining the new bone stained darkly, while the RUNX2 positively stained cells of the connective tissue were lighter stained (Figure 36b). Individual cells in the connective tissue adjacent to woven bone forming around the MB particles regularly stained positively for PCNA after six weeks. Few PCNA positive cells were observed in the cells of the connective tissues around MB particles where new bone had not formed.

MB behaved quite differently, especially up until eight weeks when MB particles were characterized by resorption and only small amounts of new bone. Large amounts of new bone around the MB graft particles were observed at 12 weeks of healing.

Other observations

Cells previously identified as osteoclasts, and other multi-nuclear cells on the surface of MB particles also stained positively using RUNX2 (Figure 39a). Mature osteocytes of the pre-existing lamellar bone did not stain brown using RUNX2 immunohistochemistry (Figure 46a).
Chapter 4 Discussion

4.1. Introduction

The pattern of healing of sites grafted with the test materials differed considerably. BO sites were characterized by early formation of bone and no obvious resorption of the BO particles. The earliest bone formed around BO particles closest to pre-existing bone. Even after 12 weeks healing, no resorption of the BO particles was observed, although small numbers of osteoclasts were found on the surface of the BO particles at all healing time points.

Early healing in MB grafted sites was characterized by large numbers of osteoclasts and obvious resorption of MB material. Large numbers of pre-osteoblasts were observed in the connective tissue surround MB particles from six weeks of healing onwards. Large numbers of cells amongst the pre-osteoblasts were also clearly proliferating where new bone was being formed.

Both materials were surrounded by bone after 12 weeks of healing in the grafted maxillary sinus site.

4.2. Differences in healing of maxillary sinus grafts using BO or MB

The surface structure of the test materials was different when viewed with SEM using medium and high magnification (Figures 16 and 17). The BO particles were covered with globular deposits that were consistent with published images of heated hydroxyapatite crystals \cite{283} and specifically BO \cite{13}. An unpublished study in which MB was heated to 500°C resulted in similar structures on MB particles \cite{15}. Images from the same study demonstrated decreased porosity of heated MB particles at very high magnification (90,000x). This observation is consistent with evidence that micro-porosity of synthetic hydroxyapatite materials decreases with increasing processing temperature \cite{284, 285}. Likewise, when two commercially available bovine-sourced
hydroxyapatite xenograft materials were compared, the material with the lowest processing temperature, BO, was found to be the most porous. Our results indicate differences in the surface structure of the two materials that may reflect the different temperatures utilized during their processing.

The source of the test materials is nearly identical, differing only in geographical location of the cattle herds. The geology of the local area may impact on the incorporation of trace elements in the hydroxyapatite such as fluoride \[286\]. There is evidence that high skeletal fluoride levels, may increase osteoclastic activity \[287\] although the levels of fluoride examined in this study were described as ‘excessive’ and the bones of rats ‘fluorotic’. In our study, only the MB particles resorbed significantly. No evidence could be located to suggest that there was excessive fluoride in the region where the animals used in the production of MB are sourced and pasture levels of fluoride in New Zealand are low \[288\]. Although the presence of trace elements in either BO or MB cannot be discounted, it seems most likely that the differences in maxillary sinus graft healing observed in our study are due to the changes in the surface porosity and structure of the hydroxyapatite crystals revealed during the SEM investigation.

This is the first report regarding the use of MB for osseous grafting so no comparison with previous histology is possible. The healing characteristics associated with MB sinus grafts was quite different to the literature concerning any other material used for sinus grafting in human or animal models. At four, six and eight weeks of healing, MB was surrounded by woven bone, fibrous connective tissue and large numbers of osteoclasts in well-defined resorption lacunae. The MB grafts were only fully surrounded by bone at 12 weeks of healing.

There was a clear temporal gradient in healing of MB grafts, similar to sites grafted with BO. The amount of bone associated with the MB particles increased over the course of the experiment. This bone was also increasingly lamellar in structure as observed with the BO grafted sites. MB particle close to pre-existing mature bone behaved similarly to BO particles, albeit surrounded by less mature and apparently smaller quantities of new bone. In this respect MB appeared to have some osteoconductive properties, although to a lesser extent than observed for BO.

In contrast, however, the appearance of new bone around MB particles distant from the pre-existing bone was different to that observed with BO. Small amounts of WB were regularly observed at considerable distance from any other bone. Within the limited scope of this study,
only a small number of sections were available for comparison, and 3D images were not available, it would appear that at least some of the new bone in this region was forming as a result of osteoinductive events occurring within the healing connective tissue matrix. Further evidence for this conclusion will be discussed in relation to the temporal changes in the distribution of osteoclasts and RUNX2 positive cells in close proximity to the MB particles in the next section.

Large numbers of osteoclasts were observed in clearly defined resorption pits on the surfaces of MB particles at four and six weeks (Figures 31b and 49b). Furthermore, particles of MB appeared noticeably smaller after 12 weeks of healing (Figure 40b). Whenever CT was in contact with the MB particles, osteoclasts were observed on the surface of the graft material, although numbers appeared to decrease over the course of the experiment. The intensity of the TRAP staining of osteoclasts associated with MB particles at four and six weeks was striking, suggesting extremely active metabolic behaviour amongst these cells. In contrast the osteoclasts did not appear to be actively proliferating, and osteoclast numbers decreased over the course of the experiment.

The experiment by Adeyemo et al. [235], in a sheep mandibular onlay graft model, made some similar observations to those recorded in this investigation. Numbers of osteoclasts decreased over the course of the experiment, especially after eight weeks. Grafts of BO protected by a BioGide® membrane were characterized by low resorption of graft material and maintenance of graft volume. By contrast, the autogenous block grafts reported in the same experiment were associated with large numbers of TRAP stained osteoclasts similar to those observed around MB grafts in the current investigation. The autogenous block grafts were significantly affected by resorption and deep resorption lacunae were depicted in the paper. New bone formation was likewise similar to that around MB particles. ‘Islands’ of new bone were located distant from other bone in the connective tissue at 12 weeks, while at the site of the pre-existing bone / graft interface a mature lamellar bone had formed.

In our study, BO behaved in a similar manner to that reported in human sinus studies [68, 241, 247]. New bone seems to grow outward from the underlying bone, with a gradient of healing extending out from the pre-existing bone into the grafted site, indicating healing by osteoconduction as has previously been described for BO grafted sites [193]. MB behaved quite differently, especially up until eight weeks, characterized by resorption of the MB and apposition of new immature bone around the graft particles. Graft volume of MB grafted sites appeared decreased at 12 weeks,
although quantification of this observation was not carried out in our study. These aspects of healing MB grafts were similar to those reported for autogenous grafts in a previous ovine model\cite{235}.

### 4.3. The experimental model

The restoration of the posterior maxilla can be compromised by insufficient bone for dental implant placement. Primary reasons for this are either resorption of the alveolus or pneumatisation of the maxillary sinus \cite{9}. The variety of different techniques used when placing implants into the region of the posterior maxilla underlines the need for understanding of the biological events surrounding graft consolidation and subsequent implant integration. The utility of a particular animal model depends on the closest possible replication of the clinical scenario being investigated. For example, the use of ligatures to induce periodontal destruction in animals may not accurately represent human periodontal disease, even with the use of genetically similar animals such as primates \cite{289}.

With respect to the maxillary sinus, few models could be said to exactly mimic the clinical situation most commonly encountered, which is a partially resorbed alveolus overlying the thin cortical bony floor of the sinus. A model system should include a combination of alveolar bone, cortical bone and a Schneiderian membrane, with sufficient space in the sinus cavity to allow clinically relevant depth of graft volume to be placed, ideally using an intra-oral approach.

The study which has best replicated this scenario is that by Hurzeler et al.(1997) \cite{118}. This investigation involved removal of molar teeth, alveolar ridge reduction and implant placement at various time points in rhesus macaques. Even this model, which reproduces many elements of human sinus grafting, was compromised by the small size of the animals. This necessitated the use of small diameter implants (2.8mm) and the placement of minimal graft volume in the small sinus space. Furthermore, current concerns with respect to the use of primates for non-essential investigations \cite{119} are likely to reduce rather than increase use of primate models.
The sheep sinus model used in the current study replicates the clinical scenario of a completely resorbed alveolus. In this situation no residual alveolar bone remains, leaving only the cortical bone of the sinus floor. The placement of a membrane over the surgical access excluded ingress of external tissues, allowing accurate modeling of sinus graft healing using a two stage technique as is recommended for the severely resorbed posterior maxilla \[16, 36\].

Further development of the current sheep model is restricted by an inability to study functional implant loading, and difficulties in obtaining primary stability necessary for simultaneous grafting and implant placement \[290\].

The size of the sinus space and the gross morphology of the sheep SM were similar to that of humans while the extra-oral approach made manipulation of the surgical field straightforward. This may have resulted in the absence of surgical complications in the current study. These observations reinforce the suggestion that the sheep maxillary sinus model is a useful tool for pre-clinical training of surgeons with respect to the Caldwell-Luc procedure \[75\].

A number of recent studies have examined the use of sheep to investigate a variety of aspects of oral and periodontal healing \[79, 90, 93, 97, 173, 235, 290-292\]. It is likely that the availability of farmed sheep in many countries, including New Zealand, combined with thorough characterization of ovine oral healing, will result in increased use of sheep as an animal model for maxillo-facial investigations, as has occurred with ovine orthopaedic models \[71, 72\].

**4.4. Experimental findings as compared to other models**

With regards to bony healing in the sheep maxillary sinus model, some differences were noted in the tissue response of BO in the current study, to those previously published. The experiment by Haas et al. (1998) \[53\], involving the simultaneous placement of implants and sinus grafting with BO in adult sheep, included histological observations after 12 weeks of healing. In their study, BO particles adjacent to the pre-existing antral wall were surrounded by a ‘wallpaper-like sheath’ of bone. The authors reported considerable variation between animals in the response to the BO grafts. They also observed graft particles distant from the pre-existing bone to be surrounded by soft tissue, rather than new bone. There were two significant methodological differences between
our experiment and the study by Haas et al. (1998) \cite{53}; their study included a titanium dental implant in the healing graft and they did not place a barrier membrane to close the window created in the antral wall prior to grafting the site. The simultaneous placement of implants may have impacted on healing in the local region, however evidence in the same experiment where implants were placed without grafting indicates that healing and bone formation was not impaired proximal to the implant. It is more likely that the failure to place a barrier and membrane and thus prevent soft tissue ingress through the graft-placement osteotomy site resulted in fibrous encapsulation of particles more distant from bone. This pattern of healing has been described in animal \cite{216} and human \cite{215} maxillary sinus grafts.

The use of a barrier membrane in conjunction with a graft of BO in an ovine model was described by Adeyemo et al. (2008), albeit surrounding a block of autogenous bone in a mandibular site \cite{235}. Soft tissue encapsulation of BO particles was only observed when the membrane was dislodged \cite{235}. Where the membrane was found to be intact at between four and 16 weeks of healing, the BO particles were surrounded by new bone that became increasingly lamellar in structure over the course of the experiment \cite{235}. The pattern of bone formation around the BO particles at 12 weeks in this experiment appeared more woven than lamellar. We found a similar response in our study after six weeks around particles distant from pre-existing bone (Figure 34a). When compared to BO grafted sites from the same region at 12 weeks the only difference observed was the more lamellar nature of the bone surrounding the BO particles in our study. This difference may be due to the large size of the block grafts utilised by Adeyemo’s group \cite{235}. The BO particles surrounding the block would have been up to 2cm away from the pre-existing bone, much further than in any of our grafts. There was a clear gradient of healing in our study within BO grafted sites, whereby BO particles more distant from pre-existing bone were surrounded by new bone at a later period than particles that were closer to pre-existing bone. The new bone around the BO particles at sites distant from pre-existing bone was also more woven in structure after the same period of healing (Figures 30a and 44a).

Adeyemo et al. (2008) \cite{235} described resorption of BO particles and confirmed the presence of osteoclasts by TRAP staining, although no images were presented. This differs from our findings as we did not find resorption of BO particles. The issue of resorption of BO particles in osseous grafts remains contentious, with reports ranging from complete resorption in a rabbit parietal defect \cite{267} to negligible resorption after 11 years in a human maxillary sinus \cite{199}. In our study
osteoclasts were still detectable at 12 weeks on the surface of some BO particles (Figure 41a and 55a). Although they were in close approximation with the BO particle surface, no overt resorption was detected. This is not to say none occurred. It is impossible to know the original outline morphology of the BO particles where osteoclasts were subsequently located. Resorption may have been occurring in broad, shallow pits. However, Adeyemo’s group describe ‘roughness’ of BO particles at 16 weeks [235], a feature that was not noted in our study. The study by Haas et al. (1998) did include an image of a multi-nucleated cell in what appeared to be a resorption pit on the surface of a BO particle [53]. These cells were only observed where new bone was not surrounding the BO particle. The longer healing time in this study of 26 weeks, and the fibrous encapsulation of these particles may account for evidence of resorption. Significant resorption of BO particles was reported by Thaller et al. (1994) in a rabbit parietal defect after 12 weeks [267]. Differences in the bony metabolism of rabbits and sheep [71, 72] and an absence of a membrane to protect the experimental defect in Thaller’s study, make comparisons with our study difficult.

Comparison of our results with those previously reported for membrane-protected, human maxillary sinus grafts, are strikingly similar. The histological appearance of the BO grafts after 12 weeks was very similar to that reported after sixteen weeks of healing in human sinus grafts in the absence of dental implants [68]. When the slightly increased rate of ovine osseous metabolism [72], is taken in to consideration, these healing periods may be considered analogous. In the report by Kim et al. (2009) [68], BO particles were regularly associated with lamellar bone interspersed with variable quantities of marrow / CT, similar to our observations after 12 weeks healing (Figure 54a).

The remaining published accounts of human sinus grafts that are comparable to our study (Table 4), report healing periods longer than 12 weeks. Regardless, they do all report residual BO surrounded by new bone in the grafted site. The only study that noted significant resorptive activity was that by Ferreira et al. (2009) [67]. It is interesting to note that these authors found that residual BO particles occupied 38% percent of the retrieved biopsies after an average of 11 months of healing. This is at the upper end of the range for residual BO in comparable studies and suggests that the resorption of BO was minimal. The image of a BO grafted site included in this paper was remarkably similar to those in our study after 12 weeks of healing (Figure 44a).
The lack of histological data from comparable healing periods in human BO grafted sinus sites to those presented in our experiment is matched by a similar deficiency for other oral sites. Bio-Oss® is commonly used for grafting of periodontal defects [293] and ridge preservation following dental extraction [31]. When BO is used in periodontal defects, the healing data is invariably reported from radiographic [294] and / or physical measurement after surgical re-entry [293, 294]. Ridge preservation is typically performed as a pre-implant procedure. Histological evidence of healing from these sites is normally gathered during implant surgery, as for implants in augmented sinuses, after an extended period of healing. Extraction sites were augmented and allowed to heal in two recent human studies investigating ridge preservation [191, 295]. Carmagnola et al. (2003) [295] described histological findings following four months of healing in a membrane-protected extraction socket. New bone was located around the BO particles however there was a large amount of connective tissue surrounding the BO material at the core of the defect. In this study, primary closure over the membrane was not achieved following surgery. Membrane exposure has been demonstrated to have a significant impact on bone formation during periodontal regeneration [296].

The results described by Carmagnola et al. (2003) differ from those reported by Artzi et al. (2000) for BO grafted extraction sites in humans after nine months of healing [191]. No barrier membrane was used to protect the coronal aspect of the grafted sites although primary soft tissue closure was achieved. The authors presented the data concerning percentages of residual BO (31%), new bone (46%) and connective tissue (21%). These histomorphometric measurements for socket preservation are very similar to the tissue proportions reported in human sinus grafts (Table 2). Furthermore, the histological image in their paper depicted BO encased in lamellar bone, identical to that from 12 weeks in our study (Figure 44a). Additionally the authors found no evidence of resorption in any area of the grafted site.

Short-term healing periods similar to our study have been reported in a dog tooth extraction site [205, 206]. After two weeks of healing the authors observed only minute amounts of new bone in BO grafted sockets compared to ungrafted controls [206]. They concluded that BO delayed early healing in this model. However, by 12 weeks of healing the same authors describe new bone surrounding the particles. This was more lamellar in the deeper portions of the socket close to pre-existing bone and very similar to the 12-week BO sites in our study. Other reports detailing
BO grafting of artificial bony defects describe similar patterns of graft healing in dogs [297] and pigs[276].

We observed a gradient of osseous consolidation in our study. A similar phenomenon was reported by Busenlechner et al. (2009) [158] after grafting Bio-Oss into mini-pig maxillary sinuses for six and 12 weeks. Graft material closer to the pre-existing sinus wall was either surrounded by bone earlier, or was more mature in appearance than graft material further away from the pre-existing bone. Similar responses have been seen in a tooth extraction model, with grafted BO particles close to the extraction socket walls and in the more apical compartment of the healed extraction site at nine months surrounded by bone that was described as more mature than that observed coronally [191]. The socket defect was not protected by a membrane in this study and it is probable that some soft tissue ingress occurred. Nevertheless the pattern of healing discussed by the authors was compatible with the observations of a gradient of healing in BO sinus grafts recorded in our study. In contrast no gradient was discernable in BO grafts in the human maxillary sinus at nine months [161]. The authors speculated that healing had advanced to the point that the regions of interest had matured sufficiently for any gradient to be obscured. This would indicate that when insufficient bone remained for primary support at an initial surgery, protocols which allow maxillary sinus grafts of BO and then a period of healing prior to implant placement are well founded.

In summary, the sheep sinus model provided a good model for the techniques of sinus wall osteotomy and SM elevation. Healing of BO in this model was similar to that of human reports of BO grafts where the same conditions were observed, i.e. lateral access sinus lift and a membrane-protected defect without simultaneous implant placement. Furthermore our histological observations are comparable with the reported healing of BO grafts in other oral sites and in other animal models. The use of sheep appears to provide an excellent alternative to primates for the study of grafting of the severely resorbed posterior maxilla.
4.4. Bone modeling and remodeling

This study reported the use of two immunohistochemical agents, PCNA and RUNX2, neither of which appear to have been reported previously in the literature in relation to sheep. Positive staining was achieved for both antigens allowing insight into osteoblastic recruitment and proliferation. In addition, the investigator utilised techniques developed in a recent study \[229\] to confirm osteoclastic activity in the healing grafts. By combining observations of these aspects of graft consolidation, considerable insight was gained into resorptive and bone formation components of maxillary sinus graft healing in our model.

4.5. Bone formation

New bone was observed around graft particles in both regions of interest at all time points. However, prior to 12 weeks of healing when graft particles were surrounded by a mixture of WB and CT, there was consistently more new bone observed around BO particles than MB particles. Similarly where the particles were surrounded by bone, the bone was more mature, or lamellar, around BO particles. These features of the healing of the two grafts indicate that healing of MB grafts is delayed in comparison to the BO grafts.

There is evidence that both the particle morphology \[298\] and the micro-porosity \[190, 299\] of a bio-material impacts upon the rate of bone formation. Furthermore the function and morphology of osteoblasts is affected by variation in the porosity of the surface substrate \[300\] including hydroxyapatite ceramics \[285, 301\]. BO particles have been shown to support the growth of osteoblasts \textit{in vitro} \[302, 303\].

RUNX2 is detectable in all stages of osteoblastic differentiation \[125\]. The author demonstrated RUNX2 staining in cells also positive for osteocalcin and osteopontin, using double staining techniques \[125\]. The generality of RUNX2 detection in osteoblast populations allows extrapolation from previous studies using markers for specific stages of osteoblastic differentiation and function, as demonstrated in an immunohistochemical characterization of NanoBone® in human jaws \[159\].
A recent study used RUNX2 as a marker for osteoblasts and pre-osteoblasts in healing rabbit sinus sites grafted with either autogenous bone, BO or βTCP \(^{100}\). After one week of healing, the authors demonstrated BO graft particles surrounded by RUNX2 immunopositive cells in a fibrous connective tissue matrix. Our experiment did not include observations from short healing time points, but RUNX2 immunohistochemical investigation at 14, 30 and 60 days \(^{100}\) detected positive cells circumjacent to the BO particles, similar to findings around BO in our study.

The RUNX2 staining of MB sections was quite dissimilar from the BO grafted sites. Initially little positive staining was detected, but by six weeks there were numerous positive stained cells surrounding the MB particles with the morphological characteristics of osteoblasts.

### 4.6. Resorption

As with osteoblasts, the function of osteoclasts has been shown to be affected by bio-material surface porosity \(^{304, 305}\). The effects of changes in porosity are far from clear, as these reports detail conflicting results. Lossdorfer et al. (2004) suggested that increased surface roughness of titanium discs reduced osteoclastic differentiation \textit{in vitro} \(^{304}\). Contrastingly, increased osteoclastic differentiation was observed on similar titanium discs in another \textit{in vitro} model \(^{305}\). Both authors suggested this may enhance osseointegration, however more research is required to ascertain the true effects, if any, and if they are relevant to the porosity of hydroxyapatite xenografts.

The regular observation of osteoclasts on the surface of BO particles was not matched by evidence of significant resorption in our study. The presence of these cells in shallow depressions on the surface of BO has previously been cited as sufficient to infer resorptive activity \(^{53}\). Resorption of sintered hydroxyapatite discs by osteoclasts was demonstrated \textit{in vitro} by Gomi (1993) \(^{306}\). Resorptive pits of up to 25µm in diameter were observed in association with TRAP positive cells after eight days \(^{306}\). The authors also reported increased osteoclastic activity of identical discs with grit roughened surfaces, possibly due to increased osteoclastic differentiation.

The size of the BO particles in the experiment ranged from 0.25mm up to 1mm in diameter. Resorption pits similar to those reported by Gomi \(^{306}\) should have been discernable within this
study if BO was similarly affected by TRAP positive cells found on BO particle surfaces, and resorption continued at the same rate throughout the experiment. Resorption of human bone may result in resorption tunnels 1 mm wide and 3 mm deep in as little as three weeks \[140\], larger than the largest particle of BO used in this study. The reason for non-resorption of BO particles remains unclear.

In our study, BO particles adjacent to pre-existing bone were nearly completely surrounded by new bone after four weeks of healing. No specimens were available prior to four weeks of healing in our study, so it is not possible to know whether osteoclasts were present prior to the formation of the new bone. When tooth extraction sites in dogs have been grafted with BO and examined after two weeks of healing, most of the BO particles had multi-nucleated cells on their surfaces suggestive of osteoclasts \[206\]. These cells were not observed in the areas where new bone was formed. At high magnification no obvious pitting or resorption was observed on the BO particles, in contrast to where similar cells were located on natural bone nearby. The authors compared their results with their previous observations in the same model after three months of healing \[205\]. In this study new bone was regularly formed around the BO particles, which were said to promote new bone formation.

**Additional observations**

Two intriguing additional observations, RUNX2 positive staining of osteoclasts and the absence of RUNX2 positive staining of mature osteocytes, were made.

With regard to RUNX2 staining of osteoclasts, this observation has been made previously \[307, 308\]. The authors cited immunolocalisation of RUNX2 in osteoclasts as evidence for phagocytosis of osteocytes and hypertrophic chondrocytes \[307\]. In this study only one third of osteoclasts were observed to stain positively for RUNX2. In our study no attempt was made to quantify the numbers of RUNX2 positively stained osteoclasts, however most osteoclasts associated with MB particles appeared to be RUNX2 positively labeled. MB particles are described as deproteinated \[210\], with no viable osteocytes. It seems unlikely that the MB particles are the source of RUNX2 in osteoclasts, although the possibility of residual protein should not be completely discounted. Both BO and MB particles regularly stained brown during immunohistochemical investigations (Figure 50a and b). This stain may indicate residual proteins in the graft materials as has been
suggested previously \([169]\), although it is more likely that it is a non-specific background stain as is frequently found in immunohistochemical investigations \([309]\).

With regard to the absence of RUNX2 staining of mature osteocytes (Figure 49a), this observation is as expected \([125, 307]\). The presence of unstained osteocytes may be useful in determining the maturity of the surrounding bone when analyzing grafted sites as well as identifying regions of pre-existing bone prior to intervention.

### 4.7. Gradient of healing

A gradient of consolidation, similar to that described by Busenlechner (2009) \([158]\), was seen for all BO specimens at all time points. The graft material closest to the pre-existing antral bony wall was the first to be associated with new bone. New bone was also normally observed to be extending out from adjacent bone, whether pre-existing or newly formed around adjacent BO particles. This would indicate that BO is primarily osteoconductive, and new bone ‘jumps’ the gaps between graft particles. Furthermore, low numbers of RUNX2 positive cells were located in the fibrous connective tissue around the BO particles and proliferation amongst these cells was seldom observed. There were no indications that would suggest that the BO material was osteoinductive.

In contrast, bone formation around MB particles was patchy. New bone was observed to have formed around particles close to pre-existing bone, however in some sites the MB particles were directly adjacent to pre-existing bone with no sign of new bone formation in the region even when new bone was forming elsewhere in the section.

In the course of osteogenesis, osteoblasts secrete many bio-active proteins which become incorporated into the mineralized matrix \([310]\). During normal bone remodeling, the action of osteoclasts upon the bone mineral releases these growth factors previously bound within the matrix, into the surrounding connective tissue \([310]\). These growth factors are responsible for induction of osteoblastic differentiation and migration up a chemotactic gradient to the site of the resorption \([311]\). Immature osteoblasts differentiate from mesenchymal precursors amongst the
tissue of the healing graft. The results from this study showed increasing intensity of staining for RUNX2 with increasing proximity of cells to the BO and MB particles. This was much more apparent in the MB sites where RUNX2 positive cells were observed deep into the surrounding connective tissue. No RUNX2 staining was observed around the MB particles where osteoclastic activity was most active. However, particles that appeared to have undergone resorption were strongly associated with RUNX2 positive cells. Sometimes resorption and bone formation was visible at different ends of the same particle (Figure 55b). These observations are consistent with those noted of healing βTCP grafts in an intra-muscular site [312]. In this study the authors observed large numbers of osteoclasts engulfing the material at four weeks of healing followed by new bone at eight weeks of healing. Formation of new bone in soft tissue sites is characteristic of osteoinductive materials [162]. The process is not well understood, but appears to be affected by the material, its porosity and the species of animal in which it is implanted [153, 313]. The results in our study suggest that in addition to being osteoconductive, MB may well induce osteoblastic differentiation and bone formation following a period of resorption as was observed by Kondo (2006). The significance of this is difficult to assess if the material behaves differently in human sites, however osteoinductive materials have significant potential benefits in osseous reconstruction. The underlying mechanism of the osteoinduction would be speculative at this stage. Possibilities include the release of bound BMPs from the MB particles [310] or the release of cytokines resulting from normal metabolism of active osteoclasts as occurs during normal bone remodeling [136].

4.8. Relationship between resorption and bone formation

There is a dynamic relationship between the activities of osteoclasts and osteoblasts. Osteoblasts dynamically regulate the activity of osteoclasts by producing OPG, which competes for binding with RANKL (also produced by osteoblasts) to RANK binding sites on osteoclasts. RANK-RANKL binding is an essential component of osteoclast differentiation and proliferation [314]. RANKL production is upregulated by osteotropic hormones and cytokines such as parathyroid hormone and IL-11. The effect of OPG binding is to down-regulate osteoclast activity. Additionally the resorptive process of osteoclasts is known to affect osteoblast activity by
releasing BMPs and other cytokines previously bound in the bone into the extracellular matrix, thus inducing osteoblastic differentiation.

In this context, the early presence of osteoblasts around osteoconductive materials may inhibit osteoclastic activity and differentiation via production of OPG. This would explain the lack of osteoclastic activity observed in our study where bone formation had already occurred around BO particles by four weeks. These sites had notably small numbers of osteoclasts, with little resorptive activity detected, as would be expected if the local osteoblasts were down-regulating osteoclastic behaviour through the expression of OPG. This scenario could be examined using specific RANKL/OPG/RANK staining as described in sheep by Baharuddin (2010) [229], although earlier healing periods than we used in our study should be considered.

The actual sequence and means of recruitment of osteoclasts and their subsequent replacement in the resorption zone by osteoblasts is unknown, and has been described as ‘one of the great enigmas of bone biology’ [136]. In our study bone was formed in sites with contrasting densities of osteoclasts. However the MB sites were characterized by large numbers of osteoclasts prior to eight weeks, and large numbers of pre-osteoblasts, as inferred by positive RUNX2 staining. Numbers of osteoclasts decreased as osteoblasts and pre-osteoblasts increased in the surrounding connective tissue. Without identification of all biologically active molecules in the healing graft, their relative abundance and how these change over time, it is not possible to fully understand the healing processes. It does appear, however, that the activity of large numbers of osteoclasts in turn induced the differentiation of large numbers of cells along the osteoblastic pathway, as normal in the ARF cycle [122].

4.9. Clinical significance

The purpose of maxillary sinus grafting is to increase the bony support available for successful implant placement. Implants may be placed following a period of graft healing (a ‘delayed’ protocol) or simultaneously during the grafting operation. Choice of protocol is usually based on the quantity of residual bone available to ensure primary implant stability. Typical of reported success is the recent study by Lee et al. (2011) where 100% of the implants placed into BO grafts after nine months of healing were successful after an average follow up period of three years [161].
Reviews of implants in augmented sinuses report success rates greater than 90% \cite{2,9}. It is clear from our study that BO grafts are characterized by in-growth of bone and little resorption. This was not the same for MB sinus grafts. New bone was observed around MB particles, however resorption of the MB material was marked. The degree of resorption was not quantified but would most likely have been associated with a reduction in graft volume. A similar reduction in graft volume can occur in maxillary sinuses augmented with autogenous bone \cite{1}. The suitability of MB for maxillary sinus grafting using a delayed placement protocol warrants further investigation.

The potential use of MB for simultaneous grafting and implant placement is intriguing. Providing there is sufficient implant stability during healing, the presence of large numbers of cells that appear to be differentiating into osteoblasts would seem to be beneficial for osseointegration. This concept has been explored in experiments where BMPs have been added to bone grafting materials \textit{in vitro} \cite{150}, in animal (sheep) sinus grafts \cite{98}.

In one study, a variety of protein-free biomaterials were combined with BMPs and compared with harvested iliac bone tissue using \textit{in vitro} cultures of human osteoblasts \cite{150}. The authors observed significantly increased rates of osteoblastic proliferation on all four biomaterials when compared both to control samples and to autogenous bone chips. The results suggested that adding BMPs to bone graft materials may enhance osseointegration. When implants were placed simultaneously with a resorbable collagen sponge loaded with human recombinant BMP (rhBMP) in an elevated sheep sinus, significant increases in bone-to-implant contact and peri-implant bone density were observed compared to implants placed in autogenous sinus grafts \cite{98}. In addition, this result was consistently observed across three implant systems (3i, Branemark and Straumann) indicating that the benefits of this approach may be general rather than specific to a particular implant surface. Furthermore, one specific period of increased bone production in this study, where bone was labeled using polychromic fluorescent markers, was identical to the period when large amounts of new bone was being laid down around MB particles in our study, i.e. eight weeks.
4.10. Confounding factors and other issues with the investigation

There were a number of issues identified during the study that may have impacted on the results.

**Insufficient numbers**

The lack of any statistical analysis, due to insufficient numbers of test animals, limits attempts to compare quantification of amounts of bone at various time points of graft healing in this study. There was insufficient time and funding to increase the number of animals included in the experiment.

**Variation in animals**

The sheep chosen for this study came from a commercial flock of cross-bred Romney ewes. It was assumed that genetically this flock would be very homogenous, thus providing good comparability between subjects. The four animals representing the eight and 12 week time points were chosen separately to the first four animals. While they should be similar genetically, they were of a slightly different age, being six months younger, and this may have resulted in different rates of healing between the two groups. The younger animals may have healed more quickly [72, 85]. However samples analysed at eight weeks were consistent with being slightly more advanced in terms of osseous healing than those observed at six weeks. These were the only animals available when the experiment commenced.

**Material processing**

It is possible that the processing and sieving, by hand, of the MB material somehow altered the surface characteristics of the material. The MB particles became difficult to handle after sieving, responding as if affected by static electricity. It is unknown whether this affected healing at any stage of the experiment, although hydrophilic implant surfaces, such as the Straumann SLA Active™, are claimed to improve cellular adhesion and peri-implant healing [315].

No attempt was made to mimic the distribution of certain size particles of BO amongst the MB grafts. It seemed that the MB material was more fragile and prone to disintegration, leading to a smaller average particle size. This was not able to be quantified during the study period.
Orientation of specimens

The decision to divide the specimen in a dorso-ventral plane was made with the intention of accessing the centre of the grafted site. The purpose of this was to allow immunohistochemical investigations to be made using the decalcified paraffin embedded material, with resin-embedded material held in reserve if the selected antigens proved unsuitable. This was a distinct possibility considering that neither of the antibodies used in our study (PCNA and RUNX2) have ever been reported previously in an ovine model in the literature. One selected antibody, bone sialoprotein (BSP), failed to react in ovine tissue with any of the techniques attempted, and a further antigen, alkaline phosphatase (ALP), was unable to be developed due to financial and temporal constraints.

In retrospect, it would appear that this impacted on the interpretation of some aspects of the study. Some sections came from the region of the osteotomy, while others only contained graft material overlying mature, undamaged antral bone. In regions close to where the bone was damaged during surgery, there was an apparent increase in osseous repair. This may have impacted on the reporting of the results. This could only be confirmed by taking serial sections from different regions of the graft site. This was not possible within the timeframe or budget of the current investigation, nor was there sufficient tissue available after division of the specimens.

Resin-embedded sections added little with regard to understanding the cellular events around healing of grafted sites. They also allowed only a small number of sections to be produced from each harvested graft site due to the significantly thicker section size and tissue lost due to the width of the cutting blade. They did, however, allow for better interpretation of the decalcified sections. Resin-embedded sections were significantly less damaged by processing than decalcified paraffin embedded sections. This allowed accurate interpretation of the artifactual spaces produced during processing of the latter.

Specimens where no graft material was located

Three specimens contained insufficient graft material to analyse, following processing and slide mounting, and were not included in the study results. All three sites were grafted with BO (157L, 163R and 164R). Initial radiographic examination of these specimens provided clear evidence
that the graft site had been harvested however following processing only occasional particles of
the grafted material could be located. The reason(s) for the lack of material in the examined
specimens are unclear, although it is not unusual in published reports of oral grafted sites[295, 199].
Two possible reasons for lack of graft material in the regions of interest were identified.

Firstly, the surgeries were not necessarily identical. The sites were accessed surgically and small
variations in technique and anatomical morphology may have resulted in more or less material
being grafted. The amount of graft material added was not absolutely quantified at any time.

Secondly, as noted above, the specimens were divided through the centre of the radiographically
identified graft site. The interpretation of the radiographic images was a subjective decision
which may not have been histologically accurate. The two halves were subsequently trimmed and
polished prior to sectioning and slide mounting. Where only small amounts of graft material were
present processing and dividing the specimens may have made detection of grafted material
problematic in the regions of interest of this study.

**Scoring system**

The scoring system used was qualitative rather than quantitative in design. Immunohistochemical staining can vary in intensity and quality, depending on diverse factors such as fixation, and processing, while the quantity of the target protein in the cell may vary according to cellular activity. In this study, the small number of test animals for each time-point rendered quantification of cell numbers redundant as no valid numerical comparison between specimens could be made.

The principal investigator scored all slides and the scoring criteria and application confirmed by
an experienced supervisor (Dr Dawn Coates). Although this system is subjective, considerable
time was spent inspecting slides to ensure reproducibility. Staining in the regions of interest was
scored on multiple occasions and no discrepancies were recorded during the study.
4.11. Conclusions and recommendations for further research

4.11.1. Conclusions

The healing of grafts of BO in the sheep maxillary sinus model was similar to that reported for BO in other animal sinus sites and human oral sites. In contrast, the healing of MB in our study was unlike that previously reported for any xenograft material. This is despite the similar origin of the two materials. Active resorption of MB particles was observed prior to 12 weeks of healing. Both materials were largely surrounded by new bone after 12 weeks of healing. The differences in the pattern of healing appear to be due to small differences in processing of the materials.

4.11.2. Characterization of healing sinus grafts

The consolidation of bone grafts is characterized by numerous cellular events. This work investigated three critical aspects of graft healing, i.e. osteoblastic differentiation, cellular proliferation and presence of osteoclasts.

The importance of the RANK/RANKL/OPG proteins in the regulation of osteoclast and osteoblast differentiation is well known. The healing of MB, in particular, was characterized by large numbers of osteoclasts and delayed bone formation, when compared to BO. Further investigation of the healing of these grafts using markers for these proteins would enable a deeper understanding of the interaction between these cells in healing sinus grafts.

The use of RUNX2 enabled identification of cells at all stages of osteoblastic differentiation. The behaviour and function of these cells will depend on exactly where they are positioned on the differentiation pathway. Analysis of tissues using specific markers for the various stages of osteoblastic function e.g. Type I collagen and osteocalcin, will allow insight into the activity of cells previously identified by RUNX2. The same issues can be investigated with respect to osteoclasts, whereby the present study was limited to positive staining for TRAP. More specific labeling of osteoclasts, and identification of pre-osteoclasts would be advantageous in understanding healing sinus grafts.

Other critical elements of graft consolidation, such as vascular development and apoptosis, remain poorly understood.
The presence of particular proteins, as detected by immunohistochemistry, does not allow for quantitative analysis. Use of tools such as qRT²-PCR will enable more detailed understanding of changes in abundance of the mRNA for these proteins.

Healing of sinuses grafted with xenograft will differ depending whether new bone is growing via osteoconduction or osteoinduction. Micro-CT imaging of whole grafts would assist in determining the three dimensional nature of healing, placing histological observations from individual slides into the context of the surrounding tissues. Micro-CT imaging will also enable assessment of any ossification gradients that may occur.

Our study was limited to the use of two sheep per time point and four time points. The healing of BO and MB at four weeks was already significantly different. The use of earlier healing time points would enable insight into the origin of osteoclasts observed. Larger numbers of test animals would enable statistical analysis of the results.

4.11.3. Investigation of the materials in different formats

Both BO and MB are available in block form. The use of block grafts for augmenting the maxillary sinus is potentially surgically more efficient and may result in decreased post-graft resorption.

Small changes in processing temperature clearly affect the surface structure of bovine derived hydroxyapatite. In turn, these changes appear to have a significant effect on the biological behaviour of the graft material. Comparison of healing using the same materials, but discretely altering their processing temperature, and thus surface characteristics, would yield further insight into this aspect of healing around deproteinated bovine bone xenografts.

The use of mixed grafts, particularly autogenous bone combined with a filler material such as xenograft or allograft is common surgical practice. In addition, numerous other biologically active materials have been added to anorganic graft materials in attempts to accelerate or optimize healing. These mixed grafts may alter bony healing significantly. Comparable studies of mixed grafts are scarce, leading to difficulties in interpretation of the clinical utility of these approaches. This model is ideal for examination of differences between various graft combinations.
MB is a particularly porous material that is resorbed following sinus grafting. These attributes may favourably impact on the healing of mixed sinus grafts. Additionally the high degree of resorption and porosity may enhance the prospects for MB for use as a carrier for growth factors and other biologically active compounds.

4.11.4. Investigations concerning dental implants

Although the sheep sinus has been demonstrated to be a good model for bone graft healing, the principal purpose for grafting the maxillary sinus is to place dental implants. Extension of this work should include implant placement and comparison of bone healing using different surgical protocols. Investigation of the differences between simultaneous grafting with implant placement and grafting with delayed implant placement are necessary to optimise clinical predictability of implants placed in augmented sinus sites.

4.11.5. Investigations concerning the osteoinductive potential of MB

Separation of osteoconductive and potentially osteoinductive events is difficult in osseous wounds. The natural healing of these sites involves differentiation and migration of osteoblasts from mesenchymal stem cells. This is the same process as occurs during osteoinduction. Examination of MB material in established animal soft tissue models, such as the mouse subcutaneous pouch, will help determine the osteoinductive potential of the material.
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Appendices

Appendix I

1. Chemical Reagents used
Molecular Grade Water (pure H\textsubscript{2}O), (Produced fresh for use from Simplicity\textsuperscript{TM} 185, Millipore Intertech, USA)

Distilled Water (dH\textsubscript{2}O), (purified via reverse osmosis unit, RiOs\textsuperscript{TM} unit, Millipore Intertech, USA)

Ethylenediamine tetraacetic acid, (EDTA) powder, Titriplex III (1.08418.1000, Merck, Germany)

Sodium Hydroxide, (NaOH) pellets, (1.06498.0500, Merck, Germany)

Xylene, C\textsubscript{6}H\textsubscript{4}(CH\textsubscript{3})\textsubscript{2}, (Ajax Finechem Pty Ltd, New Zealand)

Ethanol, C\textsubscript{2}H\textsubscript{5}OH, (High grade, Absolute Ethanol, Thermo Fisher Scientific, USA)

Methanol, CH\textsubscript{3}OH, (Emsure\textsuperscript{®}, 1.06009.6025, Merck, Germany)

Concentrated Hydrochloric Acid (HCl), (100317.2500, Merck, Germany)

Di-Ammonium Oxalate Monohydrate, (1.01190.1000, Merck, Germany)

Phosphate Buffered Saline (PBS), (Gibco\textsuperscript{™}, Invitrogen Corporation, NZ)

3, 3’ diaminobenzidine (DAB), (Sigma D3939, Sigma Aldrich, USA)

Scott’s Water, (Supplied in MedLab, made as detailed)

Haematoxylin, (Surgipath\textsuperscript{®}, Gill II Hematoxylin, Leica Microsystems, USA)

Bovine Serum Albumin (BSA), (Fraction V, Sigma Aldrich, USA)

10% Natural Buffered Formalin (NBF), (BioLab Ltd., New Zealand)

Methyl methacrylate 99% (MMA), (Sigma Aldrich, USA)
tri-sodium citrate di-hydrate (1.06448.0500, Merck, Germany)

Non-Fat Milk Powder (NFMP), (Pams, NZ)

Tween ‘80’, (Serva Electrophoresis GmbH, Heidelberg, Germany)

2. Kits
Acid Phosphatase Leukocyte Kit (387A – 1KT, Sigma-Aldrich, USA)

Vectastan Elite ABC Kit (streptococcal horse radish peroxidase (Strep HRP), Vector Laboratories Inc, USA)

3. Equipment
Piezotome™ (Satalec, Acteon, Merignac, France)

Piezotome™ SL2 and SL3 Sinus tips (Satalec, Acteon, France)

Sinus Kit, (Osstem, Korea).

Tegra-Pol, polishing machine (Struers, GmbH, ZNL Schweiz, Germany)

Silicon Carbide Paper, Grades320 - 4000 (Struers, GmbH, ZNL Schweiz, Germany)

Accutom, cutting machine, Struers (GmbH, ZNL Schweiz, Germany)

Incubating / shaking machine (Multitron®, Infors HT, Switzerland)

APES (3-aminopropyltriethoxysilane) coated slides (Lab Scientific, Inc., USA).

pH paper (Hydrion® Brilliant pH dipstick, Sigma Aldrich, USA)

pH Meter, (pH 211, Hanna Instruments, USA)

RiOs™ wall mounted Water Distillation unit, (Millipore Intertech, USA)

Simplicity™185 Water Filter, (Millipore Intertech, USA)

Dako Pen (wax pen) (Dako, Denmark)
### 4. Materials and medications used in sheep surgery

<table>
<thead>
<tr>
<th>Material or Medication</th>
<th>Purpose</th>
<th>How Used</th>
<th>Quantity</th>
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<tr>
<td>Bio Oss®</td>
<td>Investigated Graft Material</td>
<td>Packed beneath elevated sinus membrane</td>
<td>Approximately 0.25g</td>
</tr>
<tr>
<td>Moa Bone®</td>
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<td>Bio-Gide®</td>
<td>Graft Containment</td>
<td>Double layer over osteotomy site</td>
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<tr>
<td>Vicryl® (2-0 and 4-0) (coated resorbable suture )</td>
<td>Suture Material</td>
<td>Suture Surgical Site</td>
<td>One of each grade</td>
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<td>Thiopentone</td>
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<td>Intra-venous</td>
<td>20mg/kg (to effect)</td>
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<td>Halothane</td>
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<td>Inhalation</td>
<td>1-2% (to effect)</td>
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<td>Nitrous Oxide</td>
<td>General Anaesthesia</td>
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<td>Strepsin</td>
<td>Antibiotic</td>
<td>Intra-muscular</td>
<td>1/ 5ml 1 hour pre-operatively 2/ 5ml s.i.d for three days post operatively</td>
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<tr>
<td>Carprofen</td>
<td>Anti-inflammatory</td>
<td>Intra-muscular</td>
<td>5ml s.i.d for three days post operatively</td>
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<td>Mepivicaine HCl (Xylocaine® 1:20 000 with adrenaline)</td>
<td>Local anaesthetic</td>
<td>Local Infiltration</td>
<td>3 x 2.2ml cartridges around surgical site</td>
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<td>Bupivicaine HCl 5mcg/ml (Marcain®)</td>
<td>Long acting local anaesthetic</td>
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5. 0.1M Sodium Citrate Buffer Stock Solution

Ingredients

Tri-sodium citrate di-hydrate (1.06448.0500, Merck, Germany)

Molecular Grade Water (pure H₂O) (Produced fresh for use from Simplicity™185, Millipore Intertech, USA)

Concentrated Hydrochloric Acid (HCl), (100317.2500, Merck, Germany)

Equipment

pH Meter, (‘pH 211’, Hanna Instruments, USA)

Magnetic flea + stirrer

Method

14.7g tri-Sodium citrate dehydrate

500ml pure H₂O

Mix in beaker, add HCl drop by drop to adjust pH to 6.0

Sterilize at 121°C for 20 minutes.
6. Scotts Water (made and supplied by MedLab, University of Otago, Dunedin, New Zealand)

50 gram Magnesium Sulphate (BDH Chemicals, VWR International, USA)
10 gram Sodium Bicarbonate (BDH Chemicals, VWR International, USA)
5 litres dH20

7. Ethylenedinitrilotetraacetic acid (EDTA) solution
To make two litres of 10% EDTA:

Ingredients
EDTA powder, Titriplex III (Merck, 1.08418.1000, Batch K38640318 839, Germany)
NaOH pellets, (Merck, 1.06498.0500, Batch B0304598 908 ‘GR for analysis’, Germany)
Molecular Grade Water (pure H2O) (Produced fresh for use from Simplicity™185, Millipore Intertech, USA)

Equipment
pH Meter, (pH 211, Hanna Instruments, Michigan, USA)
Magnetic flea + stirrer

Method
Add 1 litre of pure H2O to a three litre glass beaker
Stir constantly
Add 200g EDTA powder
Adjust pH using NaOH pellets to 7.4
Make up volume to 2 litres with pure H2O
Sterilize at 121°C for 20 minutes.
8. Saturated Solution of Ammonium Oxalate:
To make 150mls of 4% solution

**Ingredients**

di-ammonium oxalate monohydrate (1.01190.1000, Merck, Germany)

Molecular Grade Water (pure H₂O) (Produced fresh for use from Simplicity™185, Millipore Intertech, USA)

**Equipment**

Magnetic flea + stirrer

**Method**

In fume cupboard -

Add 6 grams di-ammonium oxalate monohydrate to 150ml pure H₂O

Dissolve with stirring

9. Serum Heat Treatment

Serum used in Immunohistochemical experiments was heat treated and aliquoted prior to use.

The vial was pierced to make a small opening in the lid. The vial was then heated to 56°C for thirty minutes. The heated vial was removed and allowed to cool to room temperature before spinning at 10,000 r.p.m for ten minutes. Finally the serum was aliquoted into 500µl amounts and frozen until required for use.
Appendix II

1. Resin for embedding
Ingredients

Methyl methacrylate (M55909, Sigma Aldrich, USA)
Benzoyl peroxide (517909, Sigma Aldrich, USA)
Dibutylphthalate (524980, Sigma Aldrich, USA)
Xylene (Ajax Finechem Pty Ltd, New Zealand)

Method for MMA I

4 parts Methyl methacrylate (M55909, Sigma Aldrich, USA)
1% Benzoyl peroxide (517909, Sigma Aldrich, USA)
1 part Dibutylphthalate (524980, Sigma Aldrich, USA)

Method for MMA II

4 parts Methyl methacrylate (M55909, Sigma Aldrich, USA)
0.5% Benzoyl peroxide (517909, Sigma Aldrich, USA)
1 part Dibutylphthalate (524980, Sigma Aldrich, USA)

Method for MMA III

4 parts Methyl methacrylate (M55909, Sigma Aldrich, USA)
1% Benzoyl peroxide (517909, Sigma Aldrich, USA)
1 part Dibutylphthalate (524980, Sigma Aldrich, USA)
2. Resin embedding protocol

Immerse specimens in xylene (Ajax Finechem Pty Ltd, New Zealand) for 2 days in fume cupboard on rotating platform. Change solution twice.

Wash specimens in methyl methacrylate (MMA) monomer

Immerse specimens in MMA I for 2 days, in fume cupboard in rotating platform.

One third fill glass jars with MMA III, and place in plastic light-proof container part-filled with water. Leave without disturbing two days

Immerse specimens in MMA II for 2 days in fume cupboard in rotating platform.

Place specimen in jar with pre-set bases and cover with MMA III. Place a paper ID tag with each specimen. Orient specimen flat on set base, not touching glass jar side.

Place jar in water bath in light-tight container, at room temp in fume cupboard and leave undisturbed 2 days.
3. Staining with MacNeal's Tetrachrome / Toluidine Blue solution

Solution A

0.5 g Methylene blue (Cat. No.15943 Merck, Germany)

0.8 g Azur II (Cat. No. 9211 Merck, Germany)

0.1 g Methyl violet 2B (Cat. No. M 0527 Sigma Aldrich, USA)

250 ml Methanol (Cat. No. 1.06009.6025, Merck, Germany)

250 ml Glycerol

Mix together. Stir with magnetic stirrer until clear and no precipitate.

Leave for 12 hours @ 50°C then 3 days @ 37°C.

Solution B

0 g Toluidine blue in 100 ml distilled water + 1.0 g borax.

Combine solutions A + B:

10 ml solution A

5 ml solution B

Stir and make up to 100 ml using distilled water.

Staining protocol

Place slides in 40% ethanol in Coplin jar.

Place in ultrasonic bath for 5 minutes.

Replace ethanol with 0.1% formic acid for 5 minutes in ultrasonic bath.

Wash with tap water.

Cover section on slide with MacNeal's Tetrachrome plus Toluidine blue solution (SOLUTIONS A + B) using eyedropper for 5 minutes.

Rinse with distilled water for 5 minutes before air drying.
Appendix III

1. Specimen Decalcification Protocol
Trim specimens to minimum size, without encroaching on surgical site.

Decalcify in 10% EDTA at ratio of 1:50

Store at 4°C on gently gyrating table.

Change EDTA solution every 3 days.

Test for decalcification every three days, after ten days in solution

2. Ammonium Oxalate Test for Decalcification

Ingredients
Concentrated Hydrochloric Acid (cHCl) (Merck, 100317.2500, Germany)
Di-Ammonium Oxalate Monohydrate (Merck, 1.01190.1000, Batch A262490827, Germany)
Phosphate Buffered Saline (PBS) (Gibco™, Invitrogen Corporation, NZ)

Test Procedure
Take 5ml EDTA solution from test pot

Add cHCl until pH in range 3.2 – 3.6 (litmus paper test)

Add 5ml Ammonium oxalate

Wait 30mins – if solution remains clear = decalcification complete, transfer to PBS for paraffin-embedding. If precipitate detected, return to pot and continue decalcification as per protocol

3. Specimen Orientation
All specimens were mounted according to anatomical orientation. The cut surface where the specimen was sectioned in half was placed face down for embedding to allow sections to be taken as close to the centre of the graft as possible. The opposite side of each specimen was marked with a dye to confirm correct embedding alignment.
4. Paraffin embedding process for decalcified specimens

Automatic Processing Machine: Shandon Citadel 1000 (England, UK)

Use Program ‘A’ – Long Cycle

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Total Processing Time: 1150 mins
(19 hours 10 mins)
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### 5. Immunohistochemical Agents Used

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6. Haematoxylin and eosin routine staining
(as supplied by MedLab, University of Otago)

Reagents

Haematoxylin, (Surgipath®, Gill II Hematoxylin, Leica Microsystems, USA)

1% Eosin (Surgipath®, Leica Microsystems, USA)

Xylene (Ajax Finechem Pty Ltd, New Zealand)

Scott’s water (see reagent recipes)

Ethanol, (High grade, Absolute Ethanol, Thermo Fisher Scientific, USA)

Entellan® (Merck, Germany)

Technique

Dewax sections in xylene, 2 minutes

Transfer to 100% ethanol, 2 minutes (x4)

Wash in running tap water, 1 minute

Immerse in Gills Haematoxylin for 5 seconds

Wash in running tap water 1, minute

Immerse in Scott’s water (see reagent recipes), 1 minute

Wash in running tap water, 1 minute

Immerse in Eosin, 2 minutes

Wash in running tap water, 1 minute

Dehydrate in ethanol, 2 minutes(x4)

Immerse in xylene, 5 minutes

Mount and coverslip using Entellan®
7. Immunohistochemistry for TRAP

**Materials**

TRAP staining Kit

Acid Phosphatase Leukocyte Kit (Sigma-Aldrich Product no: 387A – 1KT)

PBS (Phosphate Buffered Saline, Gibco™, Invitrogen Corporation, NZ)

Distilled Water (dH$_2$O), (purified via reverse osmosis unit, RiOs™ unit, Millipore Intertech, USA)

EDTA decalcified, paraffin embedded tissue; cut onto APES coated slide

**Method**

De-wax and re-hydrate

Xylene 3 x 10 minutes

100% ethanol 5 min

90% ethanol 5 min

70% ethanol 5 min

50% ethanol 5 min

Distilled H$_2$O 2 min

Distilled H$_2$O 2 min

Rinse thoroughly with distilled water.

Do not allow slides to dry.

Wax pen (Dako Pen, Dako, Denmark) around sections.

Warm slides in container with distilled water at 37°C.

Keep slides in light-proof incubation box.
Make TRAP solution

Add Fast Garnet GBC Base Solution and Sodium Nitrate Solution at 1:1 ratio to a sterile tube.

Mix gently by inversion for 30 seconds.

Let stand 2 minutes. (this is solution ‘A’)

Add remaining solutions, to achieve final percentage of each by volume, while continuing to mix:

<table>
<thead>
<tr>
<th>Percentages</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>91%</td>
<td>Deionised water pre-warmed to 37°C</td>
</tr>
<tr>
<td>2%</td>
<td>Diazotized Fast Garnet GBC Solution (solution ‘A’)</td>
</tr>
<tr>
<td>1%</td>
<td>Naphthol AS-BI Phosphate Solution</td>
</tr>
<tr>
<td>4%</td>
<td>Acetate Solution</td>
</tr>
<tr>
<td>2%</td>
<td>Tartrate Solution</td>
</tr>
</tbody>
</table>

Warm tube in 37°C water bath for 5 minutes.

Make TRAP control Solution

Add Fast Garnet GBC Base Solution and Sodium Nitrate Solution at 1:1 ratio to a sterile tube.

Mix gently by inversion for 30 seconds.

Let stand 2 minutes. (this is solution ‘A’)

Add remaining solutions, to achieve final percentage of each by volume, while continuing to mix:
Percentages  Solution

93%  Deionised water pre-warmed to 37°C
2%  Diazotized Fast Garnet GBC Solution (solution ‘A’)
1%  Naphthol AS-BI Phosphate Solution
4%  Acetate Solution
0%  Tartrate Solution

Warm tube in 37°C water bath for 5 minutes.

Staining procedure

Pipette 500µl TRAP or TRAP (control) solution to each slide as per table

Incubate slides for 1 hour at 37°C in a shaker @ 20 rpm protected from light

Rinse slides thoroughly in warm tap water

Immerse for 2 seconds in haematoxylin

Rinse with tap water until clear

Rinse for 10 seconds in Scotts water

Rinse with tap water

Immerse in PBS

Add aqueous mounting agent and cover slip, seal cover slip with nail-polish
8. Immunohistochemistry for RUNX2

**Primary antibodies**

RUNX2 Rabbit polyclonal, 1.0mg/ml (NBP1-01004, Novus Bio, Littleton, USA).

Use at 10µg/ml (1:100 dilution)

Control Rabbit IgG, 400µg/ml (sc2027, Santa Cruz Biotechnology, Santa Cruz, USA)

Use at 10µg/ml (1:40 dilution)

**Secondary antibody**

Goat monoclonal anti-Rabbit H&L- F(ab)2 Fragment, Biotin (ab6012, Abcam, Cambridge, UK)

Use at 1:500 dilution

**Retrieval technique**

0.1M Sodium citrate stock solution + heat

Use at 0.01M (1:10 dilution)

**Blocking**

Goat Serum (Sigma, Cat No. sc-G9023, St Louis, USA)

**Diluant**

1% BSA (Bovine Serum Albumin, Fraction V, Sigma Aldrich, USA) / PBS (Phosphate Buffered Saline, Gibco™, Invitrogen Corporation, NZ)

Use at 10mg BSA : 1ml PBS

**Washing solution**

1% Non-fat milk Powder (Pams, NZ) / 0.1% Tween’80’(Serva Electrophoresis GmbH, Germany) / PBS

Use at 1g NFMP : 0.1ml Tween : 100ml PBS
Quenching endogenous peroxidase solution

0.3% H₂O₂ (1.07209.1000, Merck, Germany) / methanol (Emsure®, 1.06009.6025, Merck, Germany)

Use at 1ml 0.3% H₂O₂ : 100ml methanol

Other ingredients

Strep HRP (streptococcal horse radish peroxidase, Vectastan Elite ABC, Vector Laboratories, Inc, CA, USA)

DAB (3, 3’ diaminobenzidine; D3939, Sigma Aldrich, USA)

PBS (Phosphate Buffered Saline, Gibco™, Invitrogen Corporation, NZ)

Specimens

EDTA decalcified, paraffin embedded tissue; cut onto APES coated slides

Methods

DAY 1

De-wax and re-hydrate

Xylene 3 x 10 minutes (check wax has been removed)

100% ethanol 5 min (use dirty ethanol first, then clean ethanol and mark usage on clean bottle)

90% ethanol 5 min

70% ethanol 5 min

50% ethanol 5 min

Distilled H₂O 2 min

Distilled H₂O 2 min

PBS hold
**Antigen Retrieval**

Immerse in 0.01M sodium citrate buffer (pH 6.0) at 80°C for 10 min

**Wash**

PBS

2 x 5 min

Wax pen, (Dako, Denmark) around sections

**Wash**

PBS

2 x 5 min

**Blocking**

20% goat serum diluted in 1% BSA/ PBS

100 µl/ section

30 minutes at Room Temp

Remove blocking solution by tapping on to absorbent paper

**Primary antibodies**

Dilute in 1% BSA/ PBS/ 5% goat serum

(a) RUNX2 (NBP1-01004, Novus Bio, Littleton, USA)

Use at 1:100 dilution - 100 µl/ section

(b) Control Rabbit IgG, 400µg/ml (sc2027, Santa Cruz Biotechnology, Santa Cruz, USA)

Use at 1:40 dilution - 100 µl/ section

**Incubate slides overnight at 4°C in a sealed humidified container**
DAY 2

Washing

Immerse in PBS / 1% Non Fat Milk Powder / 0.1% Tween mix

3 x washes, gently agitating on rotating platform

(5 min + 20 min + 20 min)

Wash

PBS

2 x 2 min

Secondary antibody

Goat monoclonal anti-Rabbit H&L- F(ab)2 Fragment, Biotin (ab6012)

Use at 1:500 dilution - 100 µl/ section

Dilute in 1% BSA/ PBS/ 5% goat serum

Leave 1 hour at room temperature in sealed container

Wash

PBS (x2)

1 x 2 min

2 x 10 min

Quench endogenous peroxidase

Immerse in bath of 0.3% H₂O₂ / methanol for 10 min.

(Add 0.3% H₂O₂ to bath at the last minute, then add methanol).
Wash
PBS (x2)
1x 5 min
1x 10 min
Strep-HRP (from Vectastan Elite ABC)
Add 50µl of solution A to 2.5ml PBS and mix
Then add 50µl solution B and mix again.
Leave for 30 minutes at room temperature
Use 100µl/ section.
Incubate for 30 minutes in sealed container at room temperature.
Wash
PBS (x3)
1x 2 min
2 x 10 min
Develop Peroxidase
Add 2 drops of DAB to 2ml supplied buffer (Kit D3939, Dako).
Add 100µl/ section.
Leave on slide for 3 minutes
Wash
dH₂O
3x 5 minutes
Counterstain

Haematoxylin 3 seconds

Running tap water until clear

Scotts Solution 2 minutes

H$_2$O 2 minutes

100% Ethanol 30 seconds

100% Ethanol 30 seconds

100% Ethanol 30 seconds

100% Ethanol 30 seconds

100% Ethanol 30 seconds

Xylene 2x 5min

Coverslip with Entellan® (Merck, Germany)
9. Immunohistochemistry for PCNA

Primary antibodies

PCNA Mouse monoclonal anti-rat PCNA, 525µg/ml (Clone PC-10 Dako, Denmark)

Use at 5.25µg/ml (1:100 dilution)

Control Mouse monoclonal anti-rat IgG, 400µg/ml (sc 2025, Santa Cruz Biotechnology, USA)

Use at 5.25µg/ml (1:76 dilution).

Secondary antibody

Rabbit Polyclonal to Mouse H&L- F(ab)2 Fragment (Biotin), 400µg/ml (ab5761, Abcam, Cambridge, UK)

Use at 5.25µg/ml (1:76 dilution)

Retrieval technique

0.1M Sodium citrate stock solution + heat

Use at 0.01M (1:10 dilution)

Blocking

Rabbit Serum (Sigma, Cat No. sc-R9133, St Louis, USA)

Diluant

1% BSA (Bovine Serum Albumin, Fraction V, Sigma Aldrich, USA) / PBS (Phosphate Buffered Saline, Gibco™, Invitrogen Corporation, NZ)

Use at 10mg BSA : 1ml PBS

Washing Solution

1% Non-fat milk Powder (Pams, NZ) / 0.1% Tween’80’(Serva Electrophoresis GmbH, Germany) / PBS

Use at 1g NFMP : 0.1ml Tween : 100ml PBS
Quenching endogenous peroxidase solution

0.3% H$_2$O$_2$ (1.07209.1000, Merck, Germany) / methanol (Emsure®, 1.06009.6025, Merck, Germany)

Use at 1ml 0.3% H$_2$O$_2$: 100ml methanol

Other ingredients

Strep HRP (streptococcal horse radish peroxidase, Vectastan Elite ABC, Vector Laboratories, Inc, CA, USA)

DAB (3, 3’ diaminobenzidine; D3939, Sigma Aldrich, USA)

PBS (Phosphate Buffered Saline, Gibco™, Invitrogen Corporation, NZ)

Specimens

EDTA decalcified, paraffin embedded tissue; cut onto APES coated slides
Methods

DAY 1

De-wax and re-hydrate

Xylene 3 x 10 minutes (check wax has been removed)

100% ethanol 5 min (use dirty ethanol first, then clean ethanol and mark usage on clean bottle)

90% ethanol 5 min

70% ethanol 5 min

50% ethanol 5 min

Distilled H₂O 2 min

Distilled H₂O 2 min

PBS hold

Antigen Retrieval

Immerse in 0.01M sodium citrate buffer (pH 6.0) at 80°C for 10 min

Wash

PBS

2 x 5 min

Wax pen, (Dako, Denmark) around sections

Wash

PBS

2 x 5 min
**Blocking**

20% rabbit serum diluted in 1% BSA/ PBS

100 µl/ section

30 minutes at Room Temp

Remove blocking solution by tapping on to absorbent paper

**Primary antibodies**

Dilute in 1% BSA/ PBS/ 5% rabbit serum

(a) PCNA (Clone PC-10 Dako)

Use at 1:100 dilution - 100 µl/ section

(b) Control Mouse IgG (sc 2025, Santa Cruz Biotechnology)

Use at 1:76 dilution - 100 µl/ section

**Incubate slides overnight at 4°C in a sealed humidified container**

**DAY 2**

**Washing**

Immerse in PBS / 1% Non Fat Milk Powder / 0.1% Tween mix

3 x washes, gently agitating on rotating platform

(5 min + 20 min + 20 min)

**Wash**

PBS

2 x 2 min
Secondary antibody

Rabbit Polyclonal to Mouse IgG – H&L- F(ab)2 Fragment (Biotin) (ab5761)

Use at 1:400 dilution - 100 µl/ section

Dilute in 1% BSA/ PBS/ 5% rabbit serum

Leave 1 hour at room temperature in sealed container

Wash

PBS (x2)

1 x 2 min

2 x 10 min

Quench endogenous peroxidase

Immerse in bath of 0.3% H₂O₂/ methanol for 10 min.

(Add 0.3% H₂O₂ to bath at the last minute, then add methanol).

Wash

PBS (x2)

1x 5 min

1x 10 min

Strep-HRP (from Vectastan Elite ABC)

Add 50µl of solution A to 2.5ml PBS and mix

Then add 50µl solution B and mix again.

Leave for 30 minutes at room temperature

Use 100µl/ section.

Incubate for 30 minutes in sealed container at room temperature.
Wash

PBS (x3)

1x 2 min

2 x 10 min

Develop Peroxidase

Add 2 drops of DAB to 2ml supplied buffer (Kit D3939, Dako).

Add 100µl/ section.

Leave on slide for 3 minutes

Wash

dH₂O

3x 5 minutes

Counterstain

Haematoxylin 3 seconds

Running tap water until clear

Scotts Solution 2 minutes

H₂O 2 minutes

100% Ethanol 30 seconds

100% Ethanol 30 seconds

100% Ethanol 30 seconds

100% Ethanol 30 seconds

Xylene 2x 5 min

Coverslip with Entellan® (Merck, Germany)