Trabecular anisotropy and collagen fibre orientation in the mandibular condyle following experimental functional appliance treatment using sheep

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Summary
In order to study the modifying effects of functional appliances on the mechanical environment of the temporomandibular joint (TMJ), we characterised the structure of the mandibular condyle subsequent to an experimental functional appliance intervention. Eight, four-month-old, castrated male Merino sheep, were randomly allocated to experimental and control groups (n = 4 in each group). Forward mandibular displacement was induced with an intraoral appliance. The study period was 15 weeks, during which time fluorochromes were administered to all of the animals. Mid-sagittal sections of the TMJ were selected for analysis and trabecular anisotropy was estimated using bone histomorphometry. Only the experimental group demonstrated that the trabecular bone in the central condylar region was less anisotropic when compared to the subchondral region. Also, the variation in trabecular anisotropy of the central condylar region was found to be smaller in the experimental group. The collagen fibre orientation was analysed under polarised light as the proportion of the dark or bright fibres observed in regions which existed before, and regions which formed during the experiment, as determined by the fluorochrome labels. In the experimental group, more bright collagen fibres were found in the most superior region of the mandibular condyle when compared with the controls. These results suggested that the experimental functional appliances changed the orientation and pattern of the mechanical forces acting on the mandibular condyle, and possibly increased the magnitude of the lateral functional forces applied to the most superior part of the condyle during such treatments.

Keywords
Sheep mandibular condyle, growth modification, collagen fibre orientation, trabecular anisotropy

Introduction
Growth modification in the region of the human temporomandibular joint (TMJ) by the use of functional appliances is of great interest to orthodontists for the treatment of class II malocclusion. Functional appliances have been reported to achieve correction of class II discrepancy through increasing the mandibular length (17, 29, 34) and rotating the mandible (6, 35). These changes have been suggested to result both from stimulation of mandibular condylar growth beyond that which would normally occur in growing children, and redirection of condylar growth from an upward and forward vector to a more posterior orientation (36). However, the mandibular trabecular bone structural orientation and the orthopaedic mechanical stress acting on bone formation in the TMJ has not been determined.

The mandibular condyle is a growth site within the TMJ but current knowledge of the responses of the mandibular condylar tissue to functional appliances comes mainly from animal experiments. In order to mimic orthodontic treatment procedures, specific functional appliances have been used to prompt the mandible to a protrusive position in various animal species (9, 22, 26). The overall results of the subsequent bone adaptation have shown increased bone-forming activity of the mandibular condylar tissue. For example: this increased bone-forming activity has been manifested as elevated alkaline phosphatase activity and \textsuperscript{45}Ca uptake (30) and as newly-formed trabecular bone being deposited on the posterior border of the condyle and mandibular ramus (26, 32). In addition, the increased bone forming activity is not uniformly distributed among regions within the mandibular condyle (23).

The bone structure is an indicator of its mechanical properties having adapted to its mechanical environment. In trabecular bone, the anisotropy and bone volume (BV) to total tissue volume (TV) fraction (BV/TV) are of special interest, since a large proportion of the variance in the mechanical properties can be explained by these parameters (24). However, previous reports on the trabecular architecture of the mandibular condyle have not provided sufficient information on how trabecular bone structural orientation adapts to orthopaedic mechanical stress altered by a functional appliance. After experimental functional appliance treatment, a posteriorly directed rotation of the trabeculae in the mandibular condyle has been found (9). More recently, the BV/TV has been found to be lower in the mandibular condyle after the treatment despite an increased bone forming activity (23). Based on available data, functional appliance treatment seems to change the orientation of the functional forces acting on the TMJ whereas the change of the magnitude of the functional force is still uncertain. The same uncertainty regarding changes in the muscle forces following functional appliance treatment has also
been reported from the direct measurement of the masticatory muscle activities. Increased (1, 27), decreased (31, 38), and unchanged (10) muscle activities have been reported.

Since the direction of any mechanical stimulus is closely represented by the trabecular orientation in the mandibular condyle (33), any identification of trabecular anisotropy should help us to understand the characteristics of the mechanical forces that might apply to the mandibular condyle during functional appliance therapy. The first aim of the present investigation was to characterize the trabecular anisotropy in different regions within the sheep mandibular condyle following functional appliance intervention.

Study of the ultrastructure of the lamellar bone in the mandibular condyle, particularly the spatial orientation of its collagen fibres, will further elucidate the manner in which orthopaedic mechanical stress acts on bone formation in the TMJ. The lamellar bone structure is controlled during bone formation in order to adapt the bone tissue to the stresses in a particular location (25). Based on the orientation of collagen fibres in bones, one can assess the distribution of stress applied to the bone during bone growth (28).

Collagen fibres are usually observed from the birefringence in the lamellar bone when viewed under polarised light. The collagen fibres that lie in the plane of section appear bright, whereas collagen fibres perpendicular to the plane appear dark (7, 8). Based on the presumption that collagen fibre orientation in trabecular lamellar bone is regular and its orientation can be evaluated by studying its birefringence (19), we analysed the proportion of the dark and bright collagen fibres in sagittal sections of the sheep mandibular condyle. The second aim of this investigation was to characterise the lamellar ultrastructure of the mandibular condyle following functional appliance treatment in a sheep model and to draw inference regarding the mechanical environment of the TMJ.

### Materials and methods

#### Functional appliance treatment and tissue processing

The detailed experimental procedure has been reported elsewhere (22). In brief, a total of eight, four-month-old, castrated male Australian Merino sheep from the same flock were randomly assigned equally into experimental and control groups. Experimental animals received functional appliances that were analogous (22) to the Clark Twin Block which is commonly used to treat human mandibular deficiency. In effect, the appliances were cast metal splints cemented to the maxillary and mandibular teeth such that the guide planes deflected the mandible forward as the sheep tried to chew. They were designed to displace the mandible forward by four mm. Otherwise, all animals were managed in the same manner. Another four sheep from the same flock were initially assigned to a third group wearing an appliance without forward displacement of the mandible. Two sheep in this group died from reasons other than wearing the appliances. Therefore this group was excluded from further analysis (20). The total observation period was 15 weeks, during which time the fluorescent bone metabolism markers, calcine (day-1), tetracycline (day-91) and alizarin red S (day-102) labels were administered to all animals. Following sacrifice and fixation in 70% ethanol/formol saline mid-sagittal sections of the left and right TMJs were selected for histology. Tissue blocks were processed and embedded in methylmethacrylate. Serial sagittal sections were cut from the blocks using a microtome. Unstained and undecalcified 10 µm thick sections were used for fluorescence microscopy and the analysis of collagen fibre orientation. Five µm thick sections, utilising von Kossa stain, were used to measure trabecular anisotropy.

#### Postoperative care

Anaesthesia was induced in the un-premedicated sheep with a mask and maintained via an endotracheal tube with Isoflurane (2–4% in 100% oxygen) connected to a semi-closed circle system. The anaesthetised sheep was monitored for end-tidal carbon dioxide to establish adequacy of spontaneous ventilation. A pulse oximeter and an oesophageal stethoscope were used to monitor heart rate and respiratory rate. Monitoring continued until the sheep was exubated. The recovery of sheep from anaesthesia and surgery was closely monitored in the post-operative phase until the sheep was ambulatory and had begun to eat.

Up to 400 ml of Hartmans solution was given I/V intraoperatively via an indwelling catheter prior to exubation. When the sheep was breathing strongly, 0.05 mg/kg of Xylazine was given I/M, and this dose was repeated after six hours. A single dose of Fina- dyne (0.5 ml) (Flunixin HCl) was given I/M upon recovery from anaesthesia. The single dose of Finadyne was decided based on our experience where duodenal ulceration may occur in sheep when given repeated doses. Sheep were given Penicillin/Streptomycin 1ml I/M (Pen/strep, CSL Ltd) for three days post operatively. The sheep were monitored for bodyweight and food/water intakes. All

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*a* BDH Laboratory Supplies, Poole, UK.

*b* Polycut, Heidelberg, Germany.
of the animals began to eat and put on weight from day 2 post-operatively.

**The analysis of slides**

Because differences between the left and right sides of the condyle were not previously found for any variable (21–23), the data from both sides were pooled for analysis providing eight condyles in each group. Our previous results demonstrated increased mandibular condylar length in response to experimental functional appliance intervention to a similar degree reported for human studies (16). An increased bone forming activity with regional difference within the mandibular condyle (23) was also observed from these slides.

**Trabecular anisotropy**

The detailed procedure of defining the regions, within the mandibular condyle, of subchondral region and central region was also reported by Ma et al. (23). In brief, under fluorescence microscopy, the boundary of calcein label was traced on each slide. The condylar tissues located within the boundary of calcein label were considered to be pre-experimental bone (central region), whereas those condylar tissues outside the boundary of calcein label and extending towards the articular surface were considered to have formed post-experiment (subchondral region) as indicated in Fig. 3. Despite four months of bone modelling changes, the initial calcein label was easily detected.

In the trabecular bone of the mandibular condyle, an index for trabecular anisotropy (Tb.An) was calculated as the ratio of horizontal to vertical intercepts of trabeculae (Ih/Iv) using a test grid and a protocol developed by one author (N.F.) and reported previously (5). In order to test for maxima and minima of Tb.An, the protocol required the specimens of mandibular condyle to be orientated at angles ranging from 0° to 180° to the dental occlusal plane (Fig. 1). The occlusal plane was determined for each specimen at the time the specimen was harvested. The coronal process of each specimen was then dissected frontally using a low-speed diamond saw (Buehler®, USA) at approximately 120 rpm. This dissection was made perpendicular to the occlusal plane and can be observed from each section as the left borderline. The occlusal plane in each section was then determined as the plane perpendicular to this borderline. In each section, Tb.An was first measured in the central region. It was then measured in the subchondral region by sliding the section into the fixed focused field for image capturing with the orientation of the section unchanged.
The distribution of Tb.An in most of the condylar sections presents a sinusoidal shape (Fig. 2A). A sine function was used to generate a best-fit line to this distribution (NLREG version 5.3, USA). The fit of the line was evaluated by its adjusted coefficient of multiple determination (Ra2). If Ra2 was more than 0.80, the best-fit line was accepted and the minimum and maximum of Tb.An were calculated based on the best-fit line. If Ra2 was less than 0.80, the distribution of Tb.An in that section was considered as not sinusoidal. Therefore, that best-fit line was rejected and that section was excluded from the analysis. This occurred in one control sample and was due to technical errors in tissue preparation providing sub optimal histology for Tb.An analysis.

According to Biewener et al. (5), the angle at which the maximum Tb.An occurred indicated the principal alignment of trabeculae. This orientation was also deemed to represent the orientation of the mandibular compressive force. This angle was called the angle of principal compressive trabecular alignment (C2 [°]). This angle is essentially perpendicular to the articular surface. The angle at which the minimum Tb.An occurred indicated the orientation along which trabeculae were least aligned. Here the orientation was deemed to represent the orientation of the mandibular tensile force and, therefore, this angle was called the angle of principal tensile trabecular alignment (T2 [°]). It was approximately tangential to the articular surface. Minimum, maximum, C2, T2 and the angles between C2 and T2 were recorded both in the central region and the subcondral region. A schematic representation is presented in Fig. 2B.

All the measurements were obtained by using a computerised image analysis system in conjunction with Microsoft Excel 2000 programmed to deal with rotation of the specimen.

Three sections from each mandibular condyle with a minimal separation between each section of 270 µm were measured. Average values derived from these three sections were both calculated for comparison between experimental and the control groups and within the condyle between the central and subchondral regions. The reproducibility of bone histomorphometry has been extensively studied (11, 12, 37) and these standard procedures to determine reproducibility were followed in our study.

Collagen fibre orientation

The collagen fibre orientation was analysed in 8 different 1.85 x 1.8 mm² areas within the condyle. In the central condylar region, three areas were selected from the most dorso-posterior area (VI), the most dorsal area (VIII) and the ventro-posterior area (VII). In the subcondylar condylar region, five areas were selected from the most dorso-posterior area (I), the most dorsal area (II), anterior area (III), and two areas from the ventral region (IV and V). While the analysis was performed, the slides were kept at standard position with the border line set as vertical.

The polariser was installed at a standard position in the microscope throughout the experiment. The images of each area were captured under polarised light and analysed using the same computerised image-analysis system as mentioned previously. Only...
trabecular bone was selected for analysis (Fig. 4). Also in the same figure 4, three levels of light intensity were observed in the trabecular bone under polarised light: bright, dark and intermediate. The collagen fibre orientation in this study was analysed by means of the proportion of the dark or the bright field of the bone within each area. Average values were calculated in each section for the three areas in the central region and the five areas in the subchondral region. Measurements were made on two sections of each mandibular condyle. The minimal separation between each section was 270 µm. Average values derived from these two sections were calculated both for comparison between experimental and the control groups and within the condyle between the central and subchondral regions.

The interpretation of birefringence of the trabecular bone in this study was made as follows. The bright areas of the trabecular bone consist of collagen fibres in the sectioning plane (sagittal plane), the dark areas consist of collagen fibres perpendicular to the sectioning plane, whereas the intermediate areas consist of collagen fibres orientated in other directions as well as newly-formed woven bone without birefringence. Only those fibres perpendicular or parallel to the sectioning plane within the lamellar bone were analysed.

### Statistical analysis

The t-test for independent samples was used to compare the means of all the variables between control and experimental groups together with Levene’s test for equality of variance. The t-test for related samples was used to compare the means of Tb.An between the central region and the subchondral region either in the control group or in the experimental group. The SPSS software package was used for all analyses. A p value < 0.05 indicated statistical significance.

### Table 1  Mean and standard deviation (in brackets) of trabecular bone anisotropy (Tb.An) in the central and subchondral regions in the control and experimental groups.

<table>
<thead>
<tr>
<th></th>
<th>Control group (N=7)</th>
<th>Experimental group (N=8)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Central region</td>
<td>Subchondral region</td>
</tr>
<tr>
<td>T_⊥ (°)</td>
<td>44.83 (0.59)</td>
<td>45.70 (1.86)</td>
</tr>
<tr>
<td>C_∥ - T_⊥ (°)</td>
<td>135.04 (3.13)</td>
<td>137.11 (5.59)</td>
</tr>
<tr>
<td>Minima (#)</td>
<td>0.59 (0.19)</td>
<td>0.50 (0.22)</td>
</tr>
<tr>
<td>Maxima (#)</td>
<td>1.06 (0.35)</td>
<td>0.95 (0.46)</td>
</tr>
</tbody>
</table>

* indicates a significant difference within each group between the subchondral and the central region revealed by t-test for paired samples.
* indicates a significant difference between the variance in the experimental group when compared with the variance in the control group in the subchondral region or the central region revealed by Levene’s test for equal variance.

### Results

#### Trabecular anisotropy

The distribution of Tb.An was found to be sinusoidal in all of the eight condyles in the experimental group and in seven of the eight condyles in the control group. One condyle in the control group was excluded from analysis because the distribution of Tb.An was not sinusoidal.

For the trabecular bone, T_⊥, C_∥, the angle between T_⊥ and C_∥ and the maxima and the minima of trabecular anisotropy in control and experimental groups are given (Table 1). Significant differences were not detected between the control and the experimental groups. However, the variation of both the minima and the maxima of Tb.An was found to be smaller in the experimental group when compared with the control group as revealed by Levene’s test for equality of variance. Furthermore, the trabecular bone in the central region was found to be less anisotropic when subjected to a t-test for related samples in the experimental group as the result of increased minima of Tb.An,
whereas the maxima of Tb.An remained unchanged (Fig. 5). No such difference was found in the control group.

**Collagen fibre orientation**

All of the eight condyles were included in the analysis. Between the control and the experimental groups, the proportion of the dark fibres in the eight analysed areas was not found to be different. The proportion of the bright fibres, however, was significantly higher in the most dorsal area (area II) in the experimental group, whereas no significant difference between the control and the experimental group was found for the other seven areas in the subchondral region or the central region (Table 2).

Less dark fibres were found in the subchondral region than in the central region. This difference was observed both in the control and in the experimental group. Interestingly, such a difference was not observed for the proportion of bright fibres either in the control group or in the experimental group (Table 3).

The reproducibility was high since significant difference between the repeated histomorphometric intra-observer measurements was not detected by paired t-tests.

### Discussion

#### Trabecular bone alignment in the mandibular condyle

The minimum Tb.An at the angle $T_\alpha$ was found to be larger in the experimental central region when compared with that in the subchondral region. Such a difference was not found in the control group. This orientation represents the nominal orientation of the tensile force, which is approximately tangential to the articular surface. One interpretation of this result indicates that trabeculae were more strongly aligned along this direction. Such a result suggests an increased mechanical force tangential to the articular surface.

A significant difference was not identified for $C_\alpha$, which implies that the orientation of the trabecular alignment was the same in the subchondral region as that in the central region. This suggests that the deemed compressive strain orientation is the same in both regions. An interpretation lies in the findings from bone remodelling simulations in idealised, concavely incongruous model joints. Eckstein and colleagues noted that the distribution of subchondral bone density deviates considerably from the contact pressure at the joint surface. It appears to follow more closely the distribution of tensile stress (tangential to the articular surface) than that of the compressive stress due to bending (13, 18).

Although recognised by the above authors, these idealised models have inherent limitations in that they are not specifically adapted to real mechanical constraints; in particular, muscle forces and the amount of support by the surrounding trabecular and compact bone (14).

Small but statistically significant differences in the trabecular alignment, which potentially results from the nominal orientation of the tensile force in the newly-developed subchondral region and in the pre-existing central region in the experimental condyles, suggest that functional appliances change the orientation of the major functional forces. These altered functional forces could then modify the distribution and mineralisation of the bone-matrix during growth, which ultimately resulted in the change of the dimension and orientation of the mandibular condyle.

#### Collagen fibre orientation

The technical difficulty of analysing the collagen fibre orientation using polarised light is acknowledged. It has been reported that the thickness and the orientation of the slides have a significant effect on the polarised...
light transmitted through the sections (8). Efforts were made to cut the sections at the same thickness and to maintain the slides measured at a standard orientation throughout this study, as shown in Fig. 3. In addition, all the measurements were performed on two sections of each mandibular condyle. By so doing, the variation during the measurement procedures was minimized.

The ultrastructure of lamellar bone, including the spatial orientation of its collagen fibres, has a direct bearing on its mechanical properties in the cortical bone (19). Such a structure also has a direct correlation with the orientation of the mechanical forces. Early studies by Ascenzi and Bonucci (2–4) using bone birefringence found that osteons which appeared dark under polarized light were the strongest under tensile loading and those which appeared bright were the strongest under compression. A number of recent investigations have combined examinations of collagen fibre orientation and in vivo strain data from bone loading in long bone cross-sections and specific localities of cortical bone. It has been found that in vivo strain data correlated strongly with collagen fibre orientation and its loading advantages (8).

In trabecular bone, the trabecular orientation and BV/TV explain a large proportion of the variance of the mechanical properties (24). Trabecular anisotropy is related to stress distribution within the trabecular bone. Indeed, the trabecular orientation is more isotropic in the subchondral region than in the central region of the mandibular condyle (15). Therefore, it has been assumed, from a mechanical point of view, that the subchondral region of the condyle is subject to multidirectional forces, whereas mechanical forces applied to the central region are more or less unidirectional (15). In the study presented herein, the mandibular condyle was divided into central and subchondral regions. Among them, the only difference in the ultrastructure of lamellar bone between the control and the experimental groups was found in the subchondral area II where more bright collagen fibres were found following the treatment. In this area, the trabecular bone is less anisotropic than it usually is and becomes more porous (measured as a lower BV/TV) (23) after treatment with experimental functional appliances. In this circumstance, the collagen fibre orientation may have more influence on the mechanical property of the bone than it usually has and the change in collagen fibre orientation may improve the mechanical property of the condyle to sustain the functional loading induced by the experimental appliance treatment.

The direction of the chewing forces in sheep, primarily generated from the masseter muscles, is more or less vertical. Therefore, the condyle was loaded vertically in compression, with the tension antero-posteriorly as well as mediolaterally distributed. In fact, the tension could be in any direction lying in the horizontal plane. In our study, all the eight areas analysed within the condyle were in the sagittal plane. If an additional compression is in the mediolateral direction, tension could be in any direction lying in the sagittal plane. More bright collagen fibres in the sagittal plane may provide greater strength to sustain lateral forces, obviously caused by the functional appliance treatment. Area II is the most dorsal area in the subchondral region and is the closest part of the condyle to the disc and the fossa. This could explain why the difference was found only in this area. More bright collagen fibres in this area suggested that the experimental functional appliances enhanced the lateral forces.

Newly formed bone was located in the subchondral region and the pre-existing bone was located at the central region of the condyle. Within the central region, more dark collagen fibres were found in both the control and the experimental groups than that in the subchondral region. Such a difference was not found for the bright fibres. There are two possible explanations for this finding. First, the mechanical forces applied to the central region are more or less unidirectional. Dark collagen fibres (perpendicular to the sagittal plane) would be better able to sustain vertical loading created by the vertical jaw movement. The higher proportion of dark fibres in the central region indicated that the bone in the central region absorbed more vertical functional forces generated from vertical jaw movement than that in the subchondral region. The lateral functional forces, which determine the proportion of the bright fibres, have not been transmitted to the central region. Therefore, the proportion of bright collagen fibres, which are more able to sustain lateral forces, was not different between the central and the subchondral regions. Second, the birefringence of bone was observed only in lamellar bone. The data (Table 3) showed that newly-formed subchondral regions always had a smaller proportion of bone showing birefringence. This was most likely the result of the newly-formed bone in the subchondral condylar regions consisting of a smaller proportion of lamellar bone and a larger proportion of woven bone compared to the pre-existing central condylar region.

**Conclusion**

The structure of bone is sensitive to the mechanical environment. An understanding of bone deposition and alignment of the structure following the orthopaedic mechanical stress induced by experimental functional appliance treatment might reveal some of the mechanisms present during functional appliance treatment. The assumptions of the mechanical environment of the TMJ made in this study were based on the structure of the bone in the mandibular condyle. The result obtained from this study, which found more bright collagen fibres in the most dorsal part of the condyle, indicated that the magnitude of the functional forces applied to this location increased in the lateral direction. The fact that minimum Tb.An at the angle Tp was larger in the central region suggested increased mechanical forces tangential to the articular surface. These two factors acted together to achieve the dorso-posterior growth of the mandibular condyle. The results also suggested that the most dorsal part of the condyle was the key component of the mandibular condyle in the adaptive responses to functional appliance treatment.

These results indicated that the trabecular alignment and collagen fibre alignment do not present a significant difference between the control and the experimental groups in these areas, even though the growth of the condyle and the BV/TV of the
condyle were different, as has been reported previously (21, 23). Further studies are recommended to directly measure the functional forces applied to the most dorso-lateral region of the condyle during functional appliance treatment.

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