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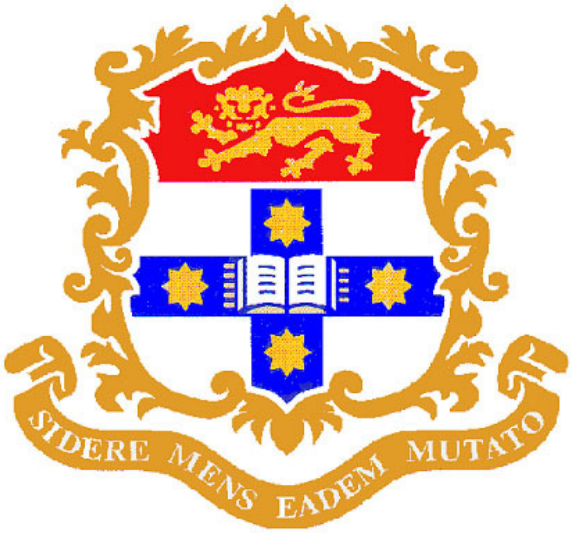
Effect of Mandibular Displacement on Condylar Cartilage Remodelling In Sprague Dawley Rats: A Micro-Structural Analysis



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1 Dedication

This Thesis is dedicated to my closest friends and family, who have travelled the long and winding path with me for the last three years. Thank you for supporting my studies and my passion as well as exhibiting never-ending tolerance and patience. A person can only be as strong as the foundations formed before them from the love of others!

2 Declaration

Candidate Certificate

This is to certify that the candidate carried out the work in this thesis in the Department of Orthodontics, University of Sydney and has not been submitted to any other University or Institution for a higher degree

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Adrian Tan

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4 Table of Contents

5 List of Figures

6 Abbreviations

2D/3D	Two/Three dimensional
SEP	Self Etch Primer (3M Unitek)
CT	Computer Tomography
Micro CT	Microscopic Computer Tomography scanner
MRI	Magnetic Resonance Imaging
NRECON	NRECON software
PG	Proteoglycan(s)
TMJ	Temporomandibular Joint
AvTvol/Tvol	(Average) Total Volume of whole condylar head comprising of bone , cartilage and periosteum
AvTcart/Tcart	(Average) Total cartilage volume of whole condylar head comprising of cartilage and periosteum
AvTvolP/TvolP	(Average) Total volume of posterior hemisection of condylar head comprising of bone, cartilage and periosteum
AvPcart/Pcart	(Average) Posterior cartilage volume of the posterior hemisection of the condylar head comprising of bone, cartilage and periosteum
Appliance	Placement of an appliance on the lower incisors of the rat.

7 Abstract

Three dimensional (3D) imaging of cartilage has always been difficult due to the inherent intermediary density between soft tissue and hard tissue in X-rays images, particularly in Micro Computer Tomography (MicroCT). Recent advances in imaging techniques have allowed for the enhancement of cartilage visualization for MicroCT use.

Aim:

The objective of this study was to provide a new insight in understanding changes in condylar cartilage, determined qualitatively and quantitatively, with normal growth and after the placement of an appliance over a 4 week period.

Materials and Methods:

Seventy Sprague Dawley rats (five weeks old) were divided into either a control group or an experimental group in which bite ramps were placed on the lower incisors at Day 0. Animals were sacrificed at Days 0, 7, 21 and 28. Right hemisections were then taken and stained with gadolinium chloride for six days before being scanned via a MicroCT unit. Condylar cartilage was digitally extracted from the scans and volumetric measurements were carried out and assessed quantitatively. Three dimensional images of the condyles were also assessed qualitatively for morphological changes between appliances and over the experimental duration. An intra-individual method error study was also carried out.

Results

Conformational changes were noted in the shape of the condyle between appliance groups and over the treatment duration. Qualitative assessment of the condyles demonstrated a reduction in size over time in all groups with a change in shape of the condylar heads. Anterior displacement of the mandible resulted in significant remodeling and distinctive shape changes that differ from both control and posterior displacement groups. Quantitative analysis demonstrated differences between control and appliance groups in regards to Total volume of the whole condylar head, Total cartilage volume, Total volume of the posterior hemisection of the condylar head and Posterior cartilage volume. The Method error study demonstrated the high reproducibility of results with a coefficient of variation of 5-13%.

Discussion

This study demonstrated a new method for analysing changes in the condylar head following orthopaedic intervention. Assessment of these changes in the condylar head can now be depicted via a three dimensional, non-destructive method. Hence, growth changes of the condylar head can now be evaluated in its totality compared to traditional methods of assessing cartilage changes sectionally via histological slices. Therefore, this provides a new avenue for improving our understanding in the changes that occur in the condylar head with growth and after intervention. It may also promote further investigations into the effects of systemic drugs on normal growth and manipulation of this important site of growth.

8 Introduction

Malocclusions are the result of a disharmony between the maxillary or mandibular dentition via its dental or skeletal components, operating either individually or as a combination of both components. The rationale for functional appliances in contemporary orthodontic practice is the utilisation of an individual's growth potential to influence the growth of the maxilla or mandible to correct jaw disharmony. If timed correctly, the use of jaw orthopaedics could correct the skeletal discrepancy, lessening the demands on orthodontic mechanics to correct the dental malocclusion with dentoalveolar compensation.

Class II malocclusions are the most frequent malocclusions seen in contemporary orthodontic practices. Typically these malocclusions make up 15-23.8% [1,2] of the population. Diagnosis of Class II malocclusions can be dental or skeletal or a combination of both. Class II malocclusions that have been a result of a skeletal disharmony in which the mandible is retrusive to a normal maxilla, are the most suitable cases for functional appliances. These types of malocclusions make up the majority of class II malocclusions.

Class III malocclusions affects about 1-5% of the general population [3-5] and are the result of genetic influences, predominantly, with potential contributions from environmental factors. Similar to Class II malocclusion, Class III malocclusions may be the result of any combination of disharmony between skeletal and dental components. Traditionally, facemask therapy has been the treatment of choice for Class III malocclusions. Facemask therapy has effects on maxillary retrusion, which represents the most common variant of Class III malocclusions, [6] as well as reciprocal effects of the mandible, reducing its protrusion. True

mandibular prognathism has been reported to occur in 20-25% of class III cases, [7] however literature examining the effects of restrictive forces to the mandible has been more limited. Chin cup therapy has been widely reported to be used in Japan for the treatment of Class III malocclusions. [8,9] Redirection of mandibular growth has been demonstrated with no significant effect on growth velocity. [8] However its useability has been questionable, as it requires long treatment duration times, patient compliance, and have a high relapse potential at the completion of treatment with catch-up growth evident. [10] Animal studies using chin cup therapy have also demonstrated significant remodelling of the condyle.[11,12]

8.1 Condylar Cartilage

Condylar cartilage of the Temporomandibular Joint (TMJ) has been described as a resilient articular-type cartilage that covers the surfaces of synovial joints. It serves to protect the underlying bone and provide lubrication for the motion of apposed surfaces. [13] To ensure normal operation of the joints, it has low friction and good load bearing properties. [14-16] Hence, the TMJ has been found to accommodate well with the compressive, shearing and tensile stresses generated by various jaw functions. [17] Apart from its articulating function, condylar cartilage also been shown to aid the flexibility of the mandibular ramus, by serving as an adjusting link between the tooth bearing jaw proper and the cranial base. [18] However its possible role in craniofacial growth is still controversial. [19]

8.1.1 Structure – Histology

8.1.1.1 Macroscopically

Previous studies have concluded that articular cartilage predominantly consists of a hydrated extracellular organic matrix and interstitial fluid, which is mostly water with relatively few cells (such as chondrocytes).[13-15,20,21] This provides cartilage with many of its structural and functional properties. The organic matrix comprises of two major macromolecular components, type II collagen and negatively charged proteoglycans (PG). [13] Due to their electronegativity (or fixed charge density [20]), PG repel each other and attract water, creating a swelling pressure that controls the compressive properties of cartilage.[14, 20] The fixed charge density, which is relatively high in cartilage, has been the source of research regarding loss of glycosaminoglycan concentration in the early detection of osteoarthritis and cartilage degeneration with Magnetic Resonance Imaging (MRI).[20] It is regarded that PG depletion results in the deterioration of cartilage mechanical properties, exposing cartilage to further damage. [14]

In normal articular cartilage, the PG concentration has been shown to be unevenly distributed and increases from superficial to deep tissue layers. [15] In the superficial zone (approximately 5-10% of cartilage thickness in humans), collagen fibrils are oriented parallel to the cartilage. In the intermediate zone, it is more randomly orientated (approximately 40-45% of cartilage thickness), and radially in the deep zone (approximately 40-45% of cartilage thickness). [15] The tissue PG content and the arrangement of the collagen network specifically affect the

mechanical characteristics of the tissue. [22,23] Thus the structural and mechanical properties are known to vary with topographical location and in different joint surfaces. [15]

8.1.1.2 Microscopically

Histologically, articular cartilage can be divided into multiple layers or zones. Differences are based on the number and orientation to the chondrocytes as well as differences in diameter and orientation of the collagen fibrils and concentration of PG. [13]

Condylar cartilage is capped with fibrous connective tissue [24,25] derived from the periosteum of the condyle. [26,27] Within the cartilage, there are undifferentiated mesenchymal (resting) cells capable of either chondrogenic or osteogenic function, depending on the environmental stimuli. [27-29] During active growth, hypertrophic chondrocytes form a major part of the cartilage. [18] In contrast to the cartilaginous growth plates, the condylar cartilage seems to lack a definite directional organization hence the calcification process appears to differ. [30]

Chondrocyte cell populations provide articular cartilage with the ability to maintain a homeostatic balance of turnover. They are highly sensitive to their environment and rely on mechanical signals, together with genetic and other environmental factors to regulate their metabolic activity. [21,31] Under physiological loading conditions, chondrocytes undergo significant changes in shape due to deformation of the cartilage extracellular matrix, suggesting that cell deformation may serve as a signal in the process of mechanical signal transduction; however this signal transduction is still unclear. [31]

8.1.1.3 Structure – Biochemical

The current state of knowledge in cartilage differentiation leaves many questions unanswered. [29] Cartilage differentiation is a highly complex phenomenon that remains incompletely understood. [29] Additionally, the sheer number of proteins that play a role in cartilage differentiation are overwhelming. These proteins can be broken down into 3 major groups: growth factors, signalling molecules and effector proteins. The first class, the growth factors, are composed of secreted proteins that initiate a cascade of events upon binding to receptors on cellular surfaces. Fibroblast growth factors, WNT proteins, Insulin-like growth factors I and II (IGF-I and IGF-II) and Hedgehog proteins fall into this category. [32,33] Signalling molecules are the intracellular proteins that convert the binding of the growth factor into a cellular response. These proteins are often growth factor specific and include membrane bound receptors and intra-nuclear transcription factors. Lastly, the effector proteins are the downstream result of growth factor signalling. These include bone morphogenic proteins, which are active in the promotion of either cartilage or bone formation. [29]

A factor complicating our understanding of cartilage differentiation is the diversity of experimental models employed in trying to elucidate its signalling pathways. Molecules found to produce or inhibit cartilage formation, as well as their subsequent molecular cascades, were first discovered in invertebrates. Although the general patterns of gene activation are probably valid, individual effects of factors can not necessarily be assumed to be identical across species. [29] Even intra-species experiments on cartilage differentiation may generate conflicting results, depending on the origin of cartilage or bone used. [29]

8.1.2 Growth of the Condylar cartilage

8.1.2.1 Embryology

Cartilage is the primary derivative of many structures in the craniofacial region, with some regions demonstrating a persistence of this embryological derived cartilage postnatally. These areas include the cranial base synchondroses, nasal septum, and the secondary cartilage of the mandibular condyle. [18] Most epiphyseal cartilages, and their associated growth plates, are sites of endochondral bone growth, allowing the elongation of the long bones in the body. On the other hand, articular cartilage growth is a result of interstitial proliferation of chondrocytes deep within the cartilage as well as cellular proliferation superficially. It is derived from a mesenchymal cell condensation, separate from Meckel's cartilage but close to the ossifying condylar process, where it eventually fusing with it. This occurs at age eight-weeks in utero and plays a role in the developing mandible early during fetal life. [18]

It has been postulated that the condylar cartilage has an innate growth potential.[34] Unlike the epiphyseal cartilages of long bones, articular cartilage does not show an innate potential for growth and bone formation, as demonstrated by transplantation experiments.[18] Thus undoubtedly, the condylar cartilage of the mandible has been the most studied of all the craniofacial cartilages, and still the most debated. [19] Its role in orthodontics has been the source of much controversy due to the potential to influence the growth of the mandible, where it is a contributor to facial appearance. However, its phylogeny demonstrates stark contrasts to that of the primary cartilages of the body, with differences seen in its organisation, calcification process and biochemical composition. [18,30]

8.1.2.2 Growth studies relating to the Condylar Cartilage

Implantation of the condylar cartilage *in vivo* [28,35] and *in vitro* [36] demonstrated that proliferation of cells could continue for some time. However, the cartilage may not be maintained, with the undifferentiated mesenchymal cells differentiating into osteoprogenitor cells. [27] It has been speculated that a feedback from the adjacent bone and articulating function may be necessary to maintain this cartilage. [36] According to experimental investigations in monkeys and rats, the condylar cartilage can be mechanically stimulated, at least at a young age. [11,37-39] The pterygoid muscle [40] and periosteum [27] have also been implicated in the growth of the condylar cartilage. Local growth defects of the mandibular ramus may occur after intervention. Condylectomies in laboratory animals [34] and bilateral condylectomies in young human patients [41] have demonstrated these growth deficiencies. Conversely, pathologic changes of the condylar region have often been associated with abnormal growth patterns of the whole mandible [24,25,42,43]. Though there is insufficient evidence to suggest that the condylar cartilage is the key to mandibular growth, [26,44] its presence is undoubtedly beneficial for normal growth of the ramus.

8.1.2.3 Normal Mandibular growth

Qualitatively, [45] mandibular growth has been described as the result of the mandible being displaced from the glenoid fossa as the soft tissue enclosing the mandible enlarges during growth and development. Resultantly, the mandible will then be displaced downwards and forwards away from the glenoid fossa. This requires the upward and backward growth of the condyle to maintain its relationship with the associated glenoid fossa in the temporal bone.

Consequently, Enlow believed that the condylar process was one of the principal sites of growth in the mandible. [45]

Quantitatively, Bjork et al [46] carried out a host of human growth studies, examining the growth of the craniofacial region. These landmark studies provided an insight in human craniofacial growth, with the use of implants placed within the facial skeleton, from which repeated cephalometric radiographs could be taken to assess the changes with growth relative to these implants. In regards to condylar growth, they found that there was significant individual variation with the growth direction not always being linear but rather having a distinct curvature. [46]

8.1.3 Growth modification – Animal studies

The effect of altered occlusion on the mandibular condylar cartilage remains unclear. [47] Functional changes in some animal models produced an increase in the width of the mandibular cartilage. [48] However other studies have demonstrated the opposite response. [49] Hence condylar growth may not necessarily be accompanied by changes in the morphology of the cartilage. [50]

The majority of studies involving condylar cartilage and mandibular growth have been by animal experimentation. Irrespective of animal model, anterior mandibular displacement has been shown to produce visible changes at the head of the condyle. [38] Furthermore, autoradiographic evidence suggests that cell division within the proliferative zone can be increased by anterior displacement. [51] Hence the evidence regarding the potential manipulation of condylar cartilage growth is still currently weak. [52] There have been

numerous studies in animals examining the effect of altered jaw function on the mandibular condylar cartilage.

8.1.3.1 Experimental *in vitro* studies

A unique feature to articular cartilage is the ability to physiologically adapt to its environment. Chondrocytes respond to mechanical signals in coordination with other environmental and genetic factors to regulate their metabolic activity. This provides the capability for articular cartilage to alter its structure and composition to meet the physical demands of the body. [21]

Petrovic and Stutzmann [40] investigated the intrinsic regulation of the condylar cartilage growth rate using organ culture plates, based on the findings of their previous experiments. It was shown that the growth rate of the condylar cartilage was controlled, not only by general, regional and local extrinsic factors but also by intrinsic mechanisms governing cell multiplication rate.

Additionally it has been revealed that chondrocytes undergo shape and volume changes in a co-ordinated manner with deformation of the cartilage tissue matrix. Guilak et al [21] examined the deformation, behaviour and viscoelastic properties of chondrocytes in articular cartilage. This was carried out via microscopic imaging and organ culture studies with the intention of characterising accurately the biophysical environment in which cartilage tissue is subjected to with physiological loads. These imaging studies identified that at the cellular level, chondrocytes undergo shape and volume changes in a co-ordinated manner with deformation

of the tissue matrix. Upon removal of any compressive forces on the tissue matrix, chondrocytes were able to recover their morphology.

On a tissue level, a compressive force causes changes in proliferation of chondrocytes and matrix synthesis in the mandibular condylar cartilage of the rat. [53] Compression of cartilage explants resulted in reductions in cell and nucleus volume, as well as changes in PG synthesis proportional to the reduction in tissue thickness. [21] When fetal rat mandibular condyles were placed under compressive loading via an organ culture system, it was found that the condylar size was not increased. [54] It has been suggested that intermittent compressive loading does not affect the size of the mandibular condyle, but when the condylar cartilage was placed under increased loading, a reduced thickness of the prechondroblastic (chondroprogenitor) and hypertrophic (maturing chondroblast) layers was observed in the cultured condyles. [54]

Three dimensional, non-linear finite-element modelling was developed for analysis of joint loading before and after the addition of condylar cartilage to the osseous mandibular condyle reconstructed from spiral computer topography data. It was concluded that the condylar cartilage absorbs considerable stresses. Hu et al [55] stated that the addition of articular cartilage primarily reduces loading of the mandibular condyle, rather than the disc and articular eminence. These findings lead to the hypothesis that the mandibular condyle more likely functions as a shock absorber than the disc.

8.1.3.2 Alterations in occlusion by incisor trimming or molar extraction

Ramirez-Yanez et al [47] examined the effect of unilateral incisor disclusion (by reducing the upper and lower incisors on one side, every second day) on cartilage thickness, mitotic activity and chondrocyte maturation and differentiation in the mandibular condylar cartilage of rats. No significant differences were observed in cartilage thickness after seven days of unilateral incisor disocclusion. However, via immunohistochemical means, unilateral incisor disclusion affects the mandibular condylar cartilage at a cellular level by increasing the mitotic activity and accelerating the maturation of chondrocytes. Chondrocyte maturation appears more accelerated on the side opposite to incisor disocclusion. [47] Teramoto et al extracted molars in rats and found changes in the collagen and PG structure in the rat condylar cartilage. [56]

8.1.3.3 Alterations in occlusion by bite raising

In vivo unilateral bite-raising has been demonstrated to increase the PG expression in the rat TMJ. [57] When the bite was raised in rats, a two-fold increase in the growth rate of the condylar cartilage was demonstrated compared to controls, which was detectable in six-month old rats within three days of appliance placement. [58] Growth was noted to be temporary and the age of the animal was a determinant of the intensity and duration of the response, as the chondrogenic cells in the cartilaginous layer of the condyle allowed for this adaptation to occur. It was noted that the enhanced growth rate starts to lessen after three weeks before returning to a normal rate after eight weeks. [58] Histologically, the cartilaginous zone thickened due to the production of cartilaginous substance with concurrent activation in

the chondrogenic zone. There was also an intensification or resumption of endochrondral bone formation and increased bone remodelling activity, indicative of renewal of growth activity at the condyle.

8.1.3.4 Change in Hard and Soft diet

Rats fed with a soft diet have been found to have a smaller mandibular condyle and thinner cartilage layer than hard-diet rats. [53] Morphologically, feeding soft diets to growing animals created changes in the condyle and ramus, which caused a shorter, narrower mandible as well as changes in its mineral content. [49] Yamada et al [49] fed weanling rats for up to eight weeks on hard and soft diets and found decreased functional force during rapid mandibular bone growth causes changes in shape. It was concluded that these changes were due to, among other things, slowed growth in the condylar cartilage. Many authors also found smaller condyles and thinner condylar cartilage in rats fed soft diets. [49,59,60] This decrease was noted in the proliferative and hypertrophic layers of the posterior region of the condyles as a result of reduced loading of the condyles.[54] It has been implicated that decreased mandibular growth in animals fed soft diets is consistent with morphologic changes evident in rats that had incisors clipped or removed, in rabbits whose mandibles were subjected to retrusive forces and in monkeys placed in intermaxillary fixations.

8.1.3.5 Anterior displacement of the mandible

The perennial work of Petrovic et al [38] on rats and bite jumping appliances has defined much of the rationale for growth modification therapies in orthodontics. They examined the effects

of bite jumping (hyperpropulsor) appliances histologically [40] on condylar cartilage and demonstrated acceleration in cartilage growth. It was concluded that a functional appliance primarily causes an increase in stem cells and prechondroblast division, an acceleration in differentiation of skeletoblasts into prechondroblasts and secondarily an increase in maturing condylar chondroblasts and an acceleration in chondroblast hypertrophy and endochondral bone growth. [61] Using tritiated thymidine labelling, they also demonstrated an increase in the growth of the condylar cartilage, depicted as an increase in the total number of cells incorporating this dye, following anterior displacement of the mandibular condyle in four week old rats. [62] Additionally, an increase in the thickness of the prechondroblastic and chondroblastic zone was also noted. Evaluating the distance between the posterior border of the condyle and the mental foramen on enlarged photographs and radiographs, Petrovic et al demonstrated an increase in the growth of the mandible. [62] Finally, it was concluded that when animals were held forward until the end of their growth period, they exhibited greater overall mandibular lengths compared to controls. Clinically, this was successfully demonstrated in humans following the application of a fixed functional appliance of mandibular retrusion during the late growth of the mandible [63]. Additionally, when the appliances were removed, no relapse occurred. [62]

Therefore, the feasibility of orthopaedically stimulating the growth of the condylar cartilage was unequivocally demonstrated by Charlier, Petrovic and Hermann-Stutzmann. [38] Hence postural hyperpropulsor (bite jumping) appliances have typically been placed in rats to examine the effects of anterior displacement of the mandible on the condylar cartilage. [52]

However, some studies suggested the converse occurs with a reduction of the hypertrophic layer in relation to the total cartilage of the condyle. Ghafari and Degroote [64] examined the

nature of condylar response to continuous propulsion in rats and found that continuous anterior and vertical displacement of the rat mandible produced an increase in area of the resting zone and accelerated ossification of the hypertrophic zone, with relative smaller and narrower hypertrophic zone relative to the total cartilaginous area.

Very similar to the study on rats, monkey studies have been the most cited of animal studies involving bite jumping appliances and its effect of mandibular growth. The presence of an appliance resulted in an alteration in the amount and direction of growth of the mandibular condyle in rapidly growing animals over 3-15 months. [64] However unlike rats, [62] which had intermittent forces applied for 8-12hours/day for periods of one, two and four weeks, McNamara et al [65] placed continuous forces in Rhesus monkeys for up to 144 weeks. McNamara et al [65] monitored changes in mandibular growth cephalometrically to investigate the long term mandibular adaptation to induced protrusive function. After 48 weeks of appliance placement, significant increases in condylar growth increment were noted and the overall mandibular length also increased. By the end of 144 weeks, it was noted that treated groups exhibited an elongation of the mandibular length by up to 5-6mm compared to controls. This constitutes an increase of 6-7% which was comparative to the 5-8% increase observed in rats. [66] McNamara et al [65] concluded that the growth of the mandible in rhesus monkeys could be increased to such an extent that the adult mandible is greater in overall length than would be achieved without experimental intervention.

Histologically and cephalometrically, craniofacial adaptations have been found following protrusive positioning of the lower jaw in nonhuman primates. [65] The condylar cartilage exhibited compensatory tissue response to experimental alterations in the postural position of the mandible. Initial changes involved hypertrophy and hyperplasia of the prechondroblastic

(resting) and chondroblastic (proliferative and hypertrophic) zones of the articular cartilage, particularly along the posterior border of the condyle. This response was maximal after six weeks. It then abated, so that by 10 weeks, only "normally occurring remodelling" was seen. [64] Woodside et al [67,68] conducted studies in monkey and found that changes not only occurred at the condylar head, but also resulted in extensive remodelling in the TMJ region with anterior relocation of the glenoid fossa. These all contributed to an improvement in the position of the mandible relative to the cranial base. Evidently, greater adaptive capacity of both the condyle and glenoid fossa was seen in juvenile animals compared to older animals. [67] The results of these studies [65,67,68] seems to indicate that the growth of the temporomandibular joint in young persons may be adaptive in nature and that the condylar cartilage was responsive to changes in function.

Ma et al [69] placed bite jumping appliances in sheep and found that in control groups, the condylar cartilage was thinnest in the anterior region and gradually thickened posteriorly. In experimental animals, anterior thickening of the condylar cartilage was demonstrated. Kantomaa [70] surgically fixated the cranial sutures of rabbits, resulting in the posterior displacement of the glenoid fossa and also demonstrated this thickening of the anterior portion of the condylar cartilage. They contributed this to the loss of articular function of the anterior condylar surface as the glenoid fossa migrates posteriorly.

8.1.3.6 Posterior displacement of the mandible

Conversely, increasing the load on the condyle by applying pressure in a posterior-superior direction via a chin cup causes a decrease in growth. The inhibition of chondrocytes growth also results in the remodelling of the TMJ structures.[11,58,71]

Like other articular cartilages of the body, the condylar cartilage is mainly a load bearing structure designed to protect against biomechanical stresses. The thickness of articular cartilage has long been suspected to be subjected to functional adaptation. [72] If non physiological stresses are applied, the adverse effects on the mechanical function of the TMJ causes remodelling of the TMJ. The resultant changes are seen as histological alterations and a decrease in condylar head volume. [72]

Janzen and Bluher [11] demonstrated retardation of condylar growth, seen as a thinner transitional zone in the posterior region of the condyles in monkeys. Charlier, Petrovic et al [38] also reported that wearing of the chin cup in young rats, which immobilised the mandible rather than displaced the mandible functionally posteriorly, brought about a retardation of the growth of the condylar cartilage by inhibiting the cell proliferation in the prechondroblastic (transitional) zone and by producing less chondroblasts.

Joho [71] investigated the effects of distally directed force applied extra orally to the lower molars via radiographical and histological methods of analysis. Restrictive forces on the mandible resulted in the resorptive changes to the glenoid fossa and significant resorption on the posterior aspect of the condyle, with compensatory bone deposition at the insertion of the lateral pterygoid muscle.

Hence, increased loading on the condylar cartilage results in the diminution of proliferative cells and a reduction in cartilage width and the amount of extracellular matrix. [56,72,73] Changes were noted within days of appliance placement, with cells in the proliferative layers

of the posterior regions of the condyles being diminished and exhibiting irregular cellular distributions. However, recovery was noted to occur within the tissue over time. [56,72]

Asano [73] investigated the three dimensional effects of a mandibular retractive force on the growing rat mandible and mandibular growth after the force was removed. Histologically, moderate adaptation of the condylar cartilage occurred with application of force. Furthermore, after removal of the retractive force, the mandibles of the experimental animals did not demonstrate any catch up growth behaviour.

However Folke and Stallard [37] found that when the mandible was displaced backwards by means of an oblique plate on the lower incisors, there was an increase in mitotic activity in the chondrogenic zone, which was greater in the posterior part of the condyle than more anteriorly.

The literature has suggested that posterior condylar displacement due to functional malocclusion causes dysfunctional remodelling of condylar cartilage. [72,73] The mandibular retractive force could produce overall growth retardation and transformation of growing rat mandibles, but the force had no effect on the growth behaviour after the appliance was removed. [11,73]

8.1.4 Growth Modification in Humans

Functional appliances are oral appliances used to produce orthopaedic changes by altering the influence of the muscle groups. These appliances affect the normal muscular function to change the normal sagittal or vertical position of the mandible. [2] They have been used since

the early 20th century and many different types have been proposed depending on their method of attachment (tooth or tissue borne) as well as its duration (intermittent or continuously acting). They have been used in the correction of mandibular retrusion by partially exerting an indirect mechanical stimulus on the condylar cartilage, an important growth site at the TMJ. [51] Many clinicians believe that anterior mandibular displacement produces a stimulation of condylar growth which results in permanent forward growth of the mandible. Numerous clinical investigations both for and against such a view have been reported. [52] However, the exact nature of the biological responses to this therapy is not well understood. [33]

Condylar growth contributes a significant proportion to the correction of class II malocclusions, however the correction of class II malocclusion involves the interplay of also the glenoid fossa adaptation, favourable growth, restriction to the maxillary dentition and maxilla, as well as differential eruption of the posterior segments.[2]

Randomised clinical trials (RCTs) have been used to assess the effectiveness of these appliances. However, these trials have failed to show the effectiveness of functional appliances to correct skeletal discrepancies. Instead these clinical trials have suggested that skeletal relationships were not dramatically improved with functional appliances, or the changes are deemed clinically insignificant. However, the RCTs conducted have been flawed due to issues regarding timing of treatment, with much early treated class II malocclusions not benefiting from early intervention. There are also classifications and methodology issues of class II as mentioned previously. Correcting timing of treatment is crucial for growth modification to be effective. Baccetti et al 2003 [74] stated that the ideal treatment timing was during the patient's peak pubertal growth period as determined by a reliable skeletal

maturation indicator (hand wrist or cervical vertebrae). Controlled clinical trials performed on Class II patients, treated at the pubertal growth spurt or shortly after that, have demonstrated significant effects. [75]

However systemic reviews on the effectiveness of functional appliances demonstrate minimal influence on the growth of the mandible. Instead correction occurs via a combination of different skeletal and dental components all contributing to the correction of class II malocclusions. [76] Cozza et al [77] conducted a systematic review of mandibular changes produced by functional appliances. They found that of 22 articles (4 RCT and 18 clinical controlled trials), 66% reported a clinically significant supplementary elongation of the the mandibular length as a result of overall active treatment with a functional appliance. This was significant if treatment was performed at the peak of pubertal growth.

Similarly, Toffol et al [78] conducted a systematic review of orthodpaedic treatment outcomes in Class 3 patients. The found that orthopaedic treatment of Class 3 patients with a combined facemask/rapid maxillary expansion appliance was able to produce 75% success after 5 years post-treatment. They found that the aetiology of Class 3 malocclusions is multifactorial, as an interaction of both hereditary and environmental components resulting in an interplay between the cranial base, maxilla and the mandible.

8.2 Methods of Analysis

Analysis of changes in condylar cartilage can be divided into two groups: two dimensional (2D) and three dimensional (3D) analyses. The majority of the literature has described changes of the condylar cartilage by means of 3D methods. These included radiology and histological methods. Recently, advancements in radiology have seen the development of new three dimensional radiological units, increasingly used in clinical and research applications.

8.2.1 Two Dimensional (2D) Methods of Analysis

8.2.1.1 Histological

Histological section has been the most reported method of analysing changes to the condylar cartilage as a result of intervention. This has been typically associated with the use of immunohistochemistry to identify changes within the cartilage layers. [38,47,58] Histology have been conducted with rats after unilateral incisor disclusion [47], posterior bite blocks [58], anterior displacement [38,52] and after compressive forces on the mandibular cartilage. [56] Histomorphometric analysis have also been used to assess changes [49,50]. Attempts have been made to relate histological changes to anatomical orientation. Buchner [58] attempted to orientate histological slices anatomically with the use of radiographs. Voudouris et al [50] attempted to standardise measurements of condylar cartilage by taking the 25th, 50th and 75th percentile histological slice. Measurements have typically been done via optical

measurements but Ramirez et al [47] measured histological slices via computer image analysis software.

There have been increasingly a number of studies examining the use of growth factors to influence the growth of the condylar cartilage. [79-89] These studies have improved our understanding of the cellular processes via the use of immunofluorescent imaging and histological sectioning.

Even though slice thickness varies from 5um [39, 64] to 20um [58], these methods only give a 2D representation of changes localised to that slice. There are methodology issues related to the orientation of sectioning and specimen preparation as well. This reduces the validity of these results as they are unable to assess changes on a gross morphological scale, but variations in findings may be the result of different planes of sections assessed. The use of biochemical markers to assess the activity of growth factors are also expensive. Finally, these methods often result in the destruction of the specimen due to the specimen preparation procedure.

8.2.1.2 Radiological

Radiology have also been used to assess gross changes in mandibular growth, typically via cephalometrics [50,65,90] or with the use of autoradiographic analysis. [52] These methods are also 2D and significant errors can occur with landmark designation. The use of implants and fluorescent bone markers have improved the measurement of condylar cartilage growth, however, its application to condylar cartilage has not been as successful as its application with the epiphyseal plates of long bones. [58]

Though the majority of condylar cartilage literature has been the result of 2D imaging and measurements, thus there are inherent issues with resolution, orientation and practicality. Their interpolations to 3D changes are also limited.

8.2.2 Three dimensional (3D) Methods of Analysis

8.2.2.1 Magnetic Resonance Imaging

Magnetic resonance imaging (MRI), a routine diagnostic tool in modern clinical medicine, has been described as one of the most powerful techniques in diagnostic medicine and biomechanical research. [91] MRI has many advantages as a diagnostic imaging modality. It is non-invasive, has excellent (sub-millimetre) spatial resolution and provides reproducible *in vivo* information. [92] Soft tissue contrast has been described as superb and readily yields anatomical information. [93] MRI also provides valuable information about the physiochemical state of tissues and blood flow. [91] Hence MRI has assumed a critical role in medical diagnosis. [94]

This level of success has been largely due to the non-invasive nature and inherent tissue contrast provided by MRI. This natural flexibility of MRI allows for the assessment of, not only anatomy, but also function in areas such as cardiac imaging, functional neuroimaging, diffusion- and perfusion-weighted MRI, and MRI angiography. [94] MRI perfectly depicts and differentiates the cartilage cap from the adjacent soft tissues, especially muscles, [95] even though it has intermediary contrast between soft tissue and bones. [96] Hence MR imaging

devices outperforms the quality of computer tomography (CT) images for diagnosis related to soft tissues. [96]

Magnetic resonance imaging has surpassed arthrography and CT for the evaluation of most patients requiring TMJ imaging. [97] During the late 1980s, MRI has gradually but rapidly emerged as the prime imaging modality for TMJ diagnosis. Previously, CT was also used to evaluate TMJ disorders, but inferior soft tissue resolution now makes CT the method of choice only when osseous TMJ abnormalities are of concern. [97]

Currently, CT superiorly demonstrates calcifications within the joint and along the cortical outline, whereas MRI continues to be the only method that can demonstrate expansion of the joint capsule and presence of joint effusion. [97]

The use of contrast agents, such as gadolinium compounds, has been implicated in MRI methods for monitoring the change in tissue electrical charge. Tissue electrical charge has been shown to change with loss of glycosaminoglycans, an early feature of osteoarthritis and early cartilage degradation. [20] These agents have been found to be very useful for imaging at near histological levels of living tissues in comparable times to *in vitro* assessments. [20] Hence the application of MRI for basic physiological studies has many advantages over other approaches. It is non-destructive, nontoxic, and specific for glycosaminoglycan concentration so that degradation can be followed over time in culture, at near histological resolution.

Though MRI is a vastly superior imaging technique for clinical medicine, its use in small animals is questionable due to their small size. Also the resolution capable via MicroCT is vastly superior to MRI.

8.2.2.2 Computer Tomography (CT)

Images generated by x-ray axial CT are considered of high quality for examination of low contrast resolution. [96] As mentioned previously, the newer and considerably more expensive MRI devices outperforms CT images for diagnosis related to soft tissue, where cartilage is an intermediate structure between a soft tissue and bones. [96] Hence the quality of TMJ examination on CT has been found to be insufficient for diagnosis of cartilaginous structures. [96]

The first reported use of a gadolinium-based media as an X-ray contrast agent in humans occurred by accident [98] when a patient received gadopentetate for a routine "unenhanced" CT scan. Contrast agents produce image contrast by their direct absorption of exogenously administered X-rays. [94] Gadolinium is an X-ray absorber. [98] Though the use of gadolinium-containing contrast media has only been approved for use in MRI, intravenous and intra-arterial injections for radiographic examinations have also been used where it has not been designed for such applications. Nevertheless, the literature suggests that it could be used for radiography safely. [98] Fortunately, despite the inherent toxicity (nephrotoxic effect) of the gadolinium ion and the relative youth of gadolinium-based contrast agents, there has been remarkable uniformity in both efficacy and safety of the first-generation compounds, making them simple to use, relatively innocuous, and essentially interchangeable. [94]

Both CT and MRI provide important information about peculiar aspects of the cartilaginous matrix such as the shape of calcifications-ossifications and lobulated growth, septa, septal enchancement and necrotic intratumoral areas. Furthermore, CT perfectly shows the patterns of bone destruction. MRI should be considered as the most reliable imaging technique for the

locoregional staging of malignant bone tumors thanks to its spatial and contrast resolution. CT plays a major role in the characterization of most bone tumors, especially those with a cartilaginous matrix. [97, 95]

MRI has gradually but rapidly emerged as the prime imaging modality for TMJ diagnosis. [97] During the 1980s CT was also used in evaluating TMJ disorders, but inferior soft tissue resolution now makes CT the method of choice only when osseous TMJ abnormalities are of primary concern. CT has high sensitivity to calcifications and is superior to MRI in this respect. [97]

Though CT improves on conventional radiography, its use in cartilaginous diagnosis is limited and has been replaced with MRI, even with the advent of new staining medium. Similar to MRI, its use is limited to clinical evaluation and MicroCT provide higher resolutions for smaller regions of interest.

8.2.2.3 Micro Computer Tomography - Bone

Recent developments in 3D MicroCT has facilitated non-destructive assessment of cancellous bone's 3D structure. [99] This is significant compare to reconstruction from histological slices. Issues such as alignment or slice crumbling do not arise, and the biopsy remains viable for reassessment. [99] The first generation of commercially available instruments was designed for studies of *in vitro* samples such as bone biopsies [100]. However, second generation instruments are capable of obtaining images from living rodents with high spatial resolution in reasonable image acquisition times. [100] MicroCT produces grayscale images, with tissue mineral content depicted by differing gray levels. Its development has seen it capable of 3D reconstruction at

a resolution of 5-50um. MicroCT has been used to evaluate changes in trabecular subchondral bone to be identified. [101] It has also been proven to be reliable with the estimating bone parameters resembling those obtained by traditional histomorphometry and may even be used for finite element modelling. [102] Image segmentation methods have been based on simple thresholding approaches, used to segment different structures with differing densities. [92] These techniques have been applied successfully not only for the segmentation of bone or trabecular, but also some soft tissues. [92] Following segmentation, relevant and meaningful measurements can be extracted such as geometrical (surface, volume, thickness, length, directional anisotropy etc) or structural parameters for tissue characterization (contrast, texture etc). [92]

Trabecular bone microscopic architecture has been of great interest ever since Wolff's theories relating structural change to functional pressure were put forth. [103] Though traditionally, serial reconstructions of histologic sections have been used, technology has advanced with 3D methods now available. These recent advances in technology have made it possible to evaluate 3D bone structure, with MicroCT highlighting its efficiency, non-destructive, and accurate qualities. [103]

Dedrick et al [101] examined the alterations in trabecular subchondral bone measured using a MicroCT in the guinea pig hind limb model of osteoarthritis. They found that these bony changes can be found as early as histological changes in cartilage. Patel et al [103] evaluated cadaver keens with MicroCT analysis and stipulated that the structural properties of subchondral bone play a role in the degeneration of articular cartilage. Whether the trabecular changes of the bone precede or follow the changes in the cartilage is unknown, but the bony

changes are considered necessary for the progression of osteoarthritis. It has even been postulated that the bony changes may precede the cartilage degeneration.

Giesen et al [102] used MicroCT to assess the 3D architecture of the human mandibular condyle and demonstrated that cancellous bone distribution was related to stress distribution on the condyle. Higher bone volumes were noted in the superior region, adjacent to the articular surface, suggesting that these areas are related to differing load conditions.

8.2.2.4 Micro Computer Tomography - Cartilage

MicroCT imaging has the potential to allow the 3D visualization of cartilage morphology. However, cartilage intensity on a MicroCT image is weak because cartilage does not strongly attenuate X-rays. [100] Hence enhanced MicroCT is a relatively new imaging modality that has the spatial resolution to visualize and quantify morphology in small animal joints. [100] It also appears to be efficient to image soft tissues, especially when enhanced with a contrast medium. [92]

Increasingly researchers have looked at using MicroCT at other structures besides bone. Roemer et al [104] conducted a pilot study examining the *in vitro* visualisation of the rat knee joint. Though they demonstrated that it was possible to quantify cartilage thickness with the use of an intra-articular contrast medium, it was not possible to visualise intrinsic early cartilaginous changes.

The development of MRI has also concurrently led to the development of chemical contrast enhancement products called contrast media. [91] They are used primarily to improve disease

detection by increasing sensitivity and diagnostic confidence. Gadolinium, as a MRI contrast medium, is a diagnostic pharmaceutical compound containing paramagnetic metal ions that affect the MRI signal properties of surrounding tissues. [105] It also has the highest neutron absorbing ability of all the elements, thus making it such an effective contrast agent. Contrast media been increasingly used to help detect and characterize various neoplastic, inflammatory and functional abnormalities [105] with the gadolinium-based compounds being, by far, the most widely used as they are generally safe and well tolerated. With improved imaging techniques, the use of contrast media, such as gadolinium, are being justified to enhance the sensitivity, specificity and diagnostic accuracy of MRI examinations.

Recent advances in MRI contrast media technologies have filtered down to applications in MicroCT. There are limited reports of the quantitative MicroCT imaging of cartilage because the inherent intensity of cartilage in a MicroCT image is weak. Hence, most MicroCT studies have so far focused on bone.

One possibility for improving cartilage visualization with MicroCT can be discerned from the MRI literature. Kusaka et al [106] used MRI to show that the uptake of gadolinium-based products in cartilage was associated with the proteoglycan distribution. Due to the paramagnetic nature of the gadolinium ion, it was able to alter the appearance of cartilage on MRI. [100]

Many investigations have focused on gadopentate (Gd-DTPA^{2-}) because it is non toxic at doses effective for MRI and is the active ingredient in a commercially available contrast agent (Magnevist). In a seminal article by Bashir et al [20], gadopentate was shown to be taken up in cartilage in inverse concentration to the proteoglycan content. Further investigation

showed that gadopentate and MRI could measure proteoglycan depletion in the rabbit knee. [100]

While the paramagnetism of gadolinium makes it useful for MRI, the metal is also known to attenuate X-rays. A pioneering study by Cockman et al [100] examined the use of a number of gadolinium-based compounds in aiding the 3D visualisation of cartilage morphology via MicroCT imaging. After testing a number of gadolinium based compounds, the use of PG chelating agents (trypsin) and a variety of exposure times and temperatures, it was concluded that treatment with 200mM Gadolinium ion (Gd^{3+}) for 24hours at room temperature, optimised cartilage intensity on MicroCT images. Thus, improved visualisation of cartilage morphology was achieved when cartilage exposed to charged gadolinium compounds. They postulated that this may provide the possibility of measuring descriptors of cartilage morphology, such as thickness and volume, from MicroCT images. [100] Recently, Sriram et al [107] demonstrated that the mandibular condylar cartilage of mice may be digitally extracted from the condylar head via the use of MicroCT and bone specific staining media.

Quantitative measurements have been successfully conducted with this use of enhanced MicroCT. Studies have shown that there is a strong positive association between PG content and gadolinium concentration. Thus this method may be used to allow a determination of PG content. [14,100]

Though MicroCT provides the most accurate structural information, its use has only been shown to be reliable in the examination of *in vitro* and are not applicable for clinical use. [108]

8.3 Summary

Our understanding of condylar cartilage has increased immensely over the last 100 years. Much of the research concerning the cellular processes involved in the development, adaptation and manipulation of the condylar cartilage has originally been derived from the medical orthopaedic literature and applied to the dental literature. However, controversy still resides regarding the ability to manipulate condylar cartilage growth. Historically, animal studies have shown histologically and radiologically, that forces applied to the condylar cartilage result in adaptive changes. Previous studies have demonstrated an increase in condylar cartilage thickness when the condylar head is displaced posteriorly into its adjacent glenoid fossa, and inversely when the condylar head is displaced anteriorly out of its fossa. The extrapolation of these findings to human application has shown mixed results with radiographic findings, being the method of analysis. The differences found between human and animal models, as well as differences within similar animal models further preclude the controversy regarding this cartilage.

Cartilage has an inherently low intensity when imaged with conventional radiology. Consequently, research involving the condylar cartilage has often been restricted to radiological and histological-based sectioning techniques, with applications in histomorphology, immunohistochemistry and biochemical studies. These methods are destructive in nature and only give 2D data. MicroCT has been increasingly used in research due to its ability to assess structures 3D without the need to damage the sample due to histological sectioning. However, MicroCT use in the TMJ region has often been relegated to imaging of bony structures. Though previous studies have highlighted the ability to image cartilage via MicroCT, these have been conducted with bone-specific staining media.

Advances in clinical medical orthopaedic imaging of cartilaginous tissues have resulted in these methods filtrating into the field of research. Recent developments in staining technologies have seen the use of traditional MRI staining media being applied to conventional radiology for image enhancement of cartilaginous tissues.

This investigation aims to examine changes in the condylar cartilage with a non-destructive analysis. Utilising the advances in radiographic imaging, it aims to qualitatively and quantitatively measure condylar cartilage volume. This may provide a new method of analysing condylar cartilage change and provide a new insight into this controversy.

8.4 Aims of this investigation

In conclusion, current knowledge on condylar cartilage and its manipulation is still unclear. After decades of orthodontic research dedicated to animal and human studies, no firm conclusions can be made regarding the effects of orthodontic intervention on this cartilaginous interface. Also, evidence has been limited due to the errors of analyses used to depict these changes, often being linear, subjective and having questionable sensitivity. With increasing advances in biomechanical imaging, the technology currently available enables quantitative assessment to enter the third dimension (3D). Hence, this study aims to investigate changes in the condylar cartilage from a 3D perspective and determine the effects of intervention with the use of modern imaging techniques.

The aims of this investigation are to:

1. Adopt the method proposed by Cockman et al [100] and modify it for the study of the condylar cartilage of the Sprague Dawley Rat.
2. Develop an imaging protocol dedicated to image the condyle of the Sprague Dawley Rat for future applications in combined pharmacological and orthopaedic interventions. [109]
3. Validate the reproducibility of this measurement protocol via a method error study.
4. Identify dimensional changes, qualitatively and quantitatively, of the condylar cartilage in a number of scenarios:
    a. Normal growth of a five week old Female Sprague Dawley rat for four week
    b. Effects of posterior displacement (BWD) of the mandible of five week old Female Sprague Dawley rats for three weeks, relative to normal anticipated growth.

c. Effects of Anterior displacement (FWD) of the mandible of five week old Female Sprague Dawley rats for three weeks, relative to normal anticipated growth.
d. Effects of recovery of condylar growth, after appliances were removed one week prior.

The hypothesis for this study is that manipulation of the condylar head will induce certain results:

1. Anterior displacement (FWD) will result in conformational changes in the condylar head and its cartilage. This would suggest an increased production of condylar cartilage on its posterior aspect, as the condylar head is displaced out of its fossa. Hence an increase in size, relative to normal anticipated growth, is expected.
2. Posterior displacement (BWD) will result in conformational changes in the condylar head and its cartilage. This would suggest the inhibition of condylar cartilage production on it posterior aspect as the condylar head is forced into the fossa. Hence a decrease in size, relative to normal anticipated growth is expected.

Hence, this study aims to disprove the null hypothesis that orthopaedic intervention has no statistical significant effect, relative to normal anticipated growth, on the cartilage volumes associated with the whole condylar head and the posterior hemisection of the condylar head.

9 Materials and Methods

9.1 Animal Housing and management

9.1.1 Acclimatisation and loading period

Approval for this study was given by The University of New South Wales (Authority to Conduct Animal Research Project (ACEC # 07/31B) - 20th April 2007) and 70 Sprague Dawley Rats (three week old) were received and processed. The Sprague-Dawley rat is one of the most widely used animals in developmental testing, primarily because of its controlled health status, out-bred genetic background and the availability of a large historical data base for many endpoints. Current historical control databases for laboratory animals are essential to the ability to identify possible treatment-related effects and appropriate background variability. [110] The animal model, type and experimentation protocol replicated previous studies in this area. [87,111]

One consignment of 35 animals were received and randomly assigned to boxes containing four animals, with the last box containing three animals. Boxes were then randomly assigned to control or appliance groups. The second consignment of 35 animals was received two days later and the same procedure was carried out. Hence, 16 boxes of four rats and two boxes of three rats were housed together in a temperature, humidity and light (12/12 hour light/dark cycle with fluorescent light) controlled room, with cages cleaned on a weekly basis. The rats were acclimatised over the first four days and monitored daily for activity and consumption

of food. Water was available *ad libitum* for each cage. Animals were given 4ml/day/rat (hence 16ml for groups of four and 12ml for groups of three) of Nutrigel (Troy Laboratories Pty Ltd, Australia – Appendix 0) for a two week loading period. This dietary regime allowed comparisons to be made with another investigation examining the effects of Glucosamine Sulphate and Chondroitin Sulphate. [109] Nutrigel has been used on rats that underwent surgical procedures as a dietary supplement given to treat weight loss. [112] Food was checked and logged for approximate amount remaining, cleaned if necessary, and added to if sufficient food was left over or a new daily supply of Nutrigel given. Food was dispensed in a small serving dish with drinking water present to improve the consistency of the Nutrigel (Figure 1). Animals commenced the experiment according to the day in which the animals were received.

9.2 Start of experimental procedures

The study design is illustrated in Figure 2 and Figure 3.

9.2.1 Control groups

At five weeks of age, 10 animals were sacrificed by carbon dioxide asphyxiation for baseline (Day 0) controls. The remaining 30 animals were maintained as per above, before a further 10 animals were sacrificed at Day 7, 10 more at Day 21 and the final 10 animals sacrificed at Day 28, the completion of the experiment.

9.2.2 Experimental groups

At five weeks of age (Day 0), 30 animals were anaesthetised by inhalational isoflurane (1-2% with 100% oxygen) and general anaesthesia (0.01mg/kg of Temgesic in Hartmann's solution), animals were monitored for respiratory function (Figure 4). They were placed individually on a Perspex board and limbs tied to the board to minimise movement of the animal and allow access to the oral cavity. The incisors of the animals were inspected and prepared with Self etch primer (SEP - 3M Unitek, USA) placing multiple coats of SEP for 40 seconds, air thinning the layers before dental composite resin (Z100 dental composite resin carpules 3M ESPE, USA) was injected into preformed Strip Form Pediatric Composite Formers (Upper primary lateral size 1 and 2, 3M ESPE, USA; Figure 5). These crown formers were modified around the gingival aspects to reduce irritation and allow excess composite resin material to be contoured around the base of the crown formers. The crown forms were then angulated forwards as much as possible to create an anterior incline plane to force forward posturing of the mandible on closure. The crowns were cured via a dental composite resin light curing machine (Elipar™ FreeLight 2 LED Curing Light, 3M ESPE, USA) for 60 seconds. The animals were finally weighed for monitoring of weight loss post appliance placement, and allowed to recover post operatively before returning them to their housings. They maintained their diet as described above and were monitored daily.

At Day 7, 10 animals were euthanized via carbon dioxide asphyxiation and weighed for changes in weight loss. The samples were prepared as described below. A further 10 animals were euthanized on Day 21, with the remaining 10 animals, having their appliances removed via discs on Day 21 (Figure 6), examined during a recovery period without appliances until Day 28, when these animals were sacrificed (Figure 7).

At the time of sacrifice, all animals were chosen randomly from the samples present, however all attempts were made to ensure no animal was isolated in a box but itself. Occlusal jaw relationships were also assessed during the experimental period and at time of sacrifice to note changes in jaw relationships. Data was maintained in a surgical spreadsheet noting the identification method for the animal, weight at appliance placement (if applicable), weight at time of appliance removal (if applicable) and weight at sacrifice (see Appendix 15.9).

9.3 Specimen preparation

The animals were decapitated and then degloved of surrounding soft tissues (Figure 8). Care was taken to avoid disturbing the muscular and ligamentous structures associated with the temporomandibular joint, as these structures were maintained. The heads were then sectioned midsagittally by an oscillating orthopaedic surgical saw and neural tissue removed to reduce the soft tissue density of the specimen. The prepared samples were then immersed in 10% buffered formaldehyde in a single sample container.

9.4 Staining Procedure

1.31gm of 99.99% Gadolinium (III) Chloride hydrate (Sigma- Aldrich Inc, St Louis, USA – Appendix 15.2) was dissolved thoroughly in 25ml of deionised (Millipore, USA) water to make 200mM Gadolinium Chloride solution. Samples were removed from their bottles of 10% buffered formalin and rinsed in three containers of deionised (Millipore, USA) water for 60 seconds each prior to being dried and placed in the 25ml of 200mM Gadolinium Chloride.

They were then sealed with Paraffin Film Laboratory Film before being stored in a fume cabinet at room temperature and pressure for 144 hours (6 days) ± 12 hours (Figure 9).

9.5 Scanning Procedure

The SkyScan 1172 (MicroCT – SkyScan Belgium) machine was warmed up to operating temperature and the X-ray tube activated for five minutes at the desired resolution (7um) to ensure consistent readings of contrast was achieved, as assessed by the supplied histogram. The MicroCT machine was then set at the desired exposure setting (See Appendix 15.3) and a flat field reference was achieved with an empty chamber to formulate a baseline exposure. Once the flat field was achieved, the image was again assessed for consistent readings for contrast and assessed whether recalibration was required prior to commencement of scanning.

Individual samples were randomly taken from their solution and dried with Kimwipes (Kimberly-Clark, USA) to remove any excess solution, before being wrapped in paraffin film (

Figure 10 and Figure 11). The samples were then mounted on a specially constructed jig fabricated from styrofoam and electrical tape was used to secure the sample tightly before being placed in the MicroCT for scanning (Figure 12).

A preliminary image was taken to ensure that the sample was centred during is scan rotation. Once sample positioning was checked at 90, 180, 270 and 360 degrees of rotation, the scan was initiated with the following settings: 7um resolution, medium camera pixel size (2000x2000x1048) and rotational movements of 0.39 degrees for 360 degrees rotation with

average framing of two. Scans were saved as BMP format and took approximately 60 to 90 minutes to complete.

On completion of the scan, the raw images were processed in NRECON (SkyScan, Belgium) to ensure that the sample was centred in the image prior to removal.

Samples were then rinsed in three dilutions of deionised water, approximately one minute per dilution, before being return to their original sample bottles containing 10% buffered formalin and sealed with Paraffin film.

Raw scanned images were then imported into NRECON (SkyScan, Belgium) and the necessary post-alignment adjustment was made to reduce any movement in the image that may have occurred during the scan. Minimal smoothing of the image was done and the raw data was cropped to only reconstruct the condyle and the associated zygomatic ridge in a square data set. The histogram was altered to improve the contrast level, by reducing the high threshold Hounsfield Units to the beginning of the image histogram. The raw data was then reconstructed over a 90-120 minute period in BMP files for use in VGStudioMax (Volume Graphics GmhB, Germany).

9.6 3D Reconstruction

The reconstructed data was then imported into VGStudioMax and a gross evaluation of the axial slices of the sample was assessed to ensure that the image was suitable for analysis (Figure 13). An initial gross grayscale threshold was undertaken to roughly remove the noise surrounding the condyle and allow preliminary sample visualisation. Once satisfied that the

reconstructed data was correct, a maximum clipbox dimension of 600x600x900 slices was used to reduce the dataset to only the area encompassing the entire condyle through all the Z – slices (antero-posterior dimension). The co-ordinates of this defined clipbox were then noted before being re-imported. This cubic dimension was adequate in allowing sufficient information to be present so that the sample could be reorientated without the risk of eliminating important information during the rotation procedure. A gross gray-scale threshold was noted to reduce background noise and optimise sample visualisation.

All computed methods were done on a specially constructed PC (AMD Opteron Dual Core Processor with 4 gigabytes of RAM) running Windows XP 64 Bit and a 64 Bit version of VGStudioMax V1.2.

9.6.1 Orientation

The reduced 3D (600x600x900 slices) image stack was then viewed from all perspectives and the rotation tool in VGstudioMax was used to align the condyle in the following specification. Rotations were made to the closest axes, to minimise the amount of rotation of the sample.

9.6.1.1 Anterior View

The sample was orientated so that the length of the coronoid process, closest to the condylar cartilage, was viewed to be parallel to a reference plane (horizontal or vertical) in space when viewed from a superior position. When viewed from the anterior, this plane was usually a point as the reference plane was viewed along this plane. Rotations were made in increments in one plane of space only and noted (
Figure 14).

9.6.1.2 Posterior view

The sample was checked that the posterior border of the ramus was aligned in the same plane of space as the anterior view. Adjustments to the same plane of space as the Anterior View and noted.

9.6.1.3 Lateral view

The partially orientated sample was then viewed from a lateral direction and orientated so that the demarcation between the condylar cartilage (identified as the lateral pole of the condylar head) and the remaining bone on the lateral side of the sample was parallel to a reference plane (horizontal or vertical – perpendicular to the plane used for the anterior and superior view). Typically, a tangent was taken with the straightest portion of the demarcation line. Rotations were made in increments in one plane of space only and noted (
Figure 15).

9.6.1.4 Superior view

The partially orientated sample was then viewed superiorly to check that the coronoid ridge was aligned to the reference plane used in the Anterior view. This plane (horizontal or vertical) now represents a line as it is viewed from above and the sample was rotated until the coronoid ridge was parallel to this plane. Rotations were made in increments in one plane of space only and noted (
Figure 16).

9.6.1.5 Inferior view

The sample was checked by ensuring that the sample was orientated in the same plane as the Superior view, when the sample was clipped from an inferior to superior direction.

On completion of the rotational movements, all rotations were summed in their corresponding X, Y and Z axes. The sample was then rotated with the summed rotations and checked that all anatomical landmarks were satisfied.

When viewed superiorly, all condyles were aligned so that the coronoid process was parallel to the midsagittal plane. Anatomically, the condylar process of rats is typically angled towards the midsagital plane. This angle, the condylar axis, was measured in preliminary samples of Sprague Dawley Rat dry skulls to be approximately 15 degrees to the midsagittal plane (Figure 18). This arbitrary angulation was given to all orientated samples, to represent them as anatomically as possible as well to allow comparisons between samples. The final rotations were then summed and noted.

On completion of the orientation stage, the sample was re-imported into VGStudioMax with the defined clipbox, as noted previously, and then exported as a sample containing only 600x600x900 slices, named: [sample designation]_preorient.

9.7 Reorientation of data

To allow the condyle to be sectioned accurately via the clipbox tool on VGStudioMax, the data sets needed to be reorientated to the desired orientations identified previously.

RotateScanline was specifically developed by the University of Sydney (Electron Microscopy Unit) to allow the reorientation of datasets in three planes of space when the rotations required about the x, y, z axis are known. Alteration of the data sets may result in lost in definition of the images as a result of the post-image processing. A preliminary trial of this program (conducted by one of the co-authors (G. (Cohn) Shields, Electron Microscopy Unit, University of Sydney)) examine the changes in volume using this software in the rotation of a symmetrical object found that the error between non-rotated and a rotated sample (up to 25 degrees) was approximately 3%. This validated the use of this software in orientating the data.

The recently exported dataset ([sample designation]_preorient) was then imported into RotateScanline (EMU, University of Sydney) for reorientation of data sets. A maximum 600x600x900 slice sample was taken, as the software can only successfully orientate samples with total number of slices divisible by four, but also to allow sufficient data to be present so clipping of the final data set can be achieved successfully without impinging on the area of interest (See Appendix 15.5). Preliminary testing of this software identified that this data size was sufficient to allow rotation of the data without compromising the processing power of the workstation. The summed rotations were inputted into the software and the rotations were carried out simultaneously. On completion of the rotation, the orientated data set was exported as a new dataset ([sample designation]_orient).

The new dataset ([sample designation]_orient) was then imported into VGStudioMax and compared to the preorientated ([sample designation]_preorient) sample with the rotation axes inputted. The samples were superimposed over each other to ensure rotation was carried out correctly.

9.8 Segmentation and Measurement

Once the orientation sample's rotation was verified, the orientated sample was imported into VGStudioMax and the threshold value obtained at the beginning of the analysis was used. The sample was visually clipped so that the clipbox only encompassed the condylar process with minimal external noise (i.e. zygomatic arch) as possible. The following criteria were applied:

9.8.1 Maximum Linear Dimensions

9.8.1.1 Medio-laterally Dimension

The sample was clipped in a medio-lateral dimension, until the slice corresponding with the most medial and most lateral portion of the condyle was identified. The co-ordinates of these locations were noted.

9.8.1.2 Antero-posterior Dimension

The sample was clipped in an antero-posterior direction, until the slice corresponding with the most posterior portion of the condyle was identified. Anteriorly, the most anterior portion of the condylar cartilage was identified as the most anterior demarcation line (as viewed from a lateral and anterolateral view) between the condylar cartilage and adjacent bone of the coronoid process moving anterior to it. The co-ordinates of these locations were noted.

9.8.1.3 Superior-inferior Dimension

The sample was clipped in a superior-inferior direction, until the slice corresponding with the most superior portion of the condyle was identified. Inferiorly, the sample was clipped from an inferior-superior direction, until the demarcation line noted on the lateral surface was evident. The inferior surface of the lateral pole of the condylar head represented the most inferior point of the condylar head and this location was noted. Due to the irregular shape of the condyle cartilage in regards to its anterior and posterior extension, an arbitrary number of slices (180 slices → 1260um) were taken to standardise the vertical dimension of all samples. The posterior extension extends more inferior around the posterior border of the ascending ramus relative to its anterior extension, requiring the need to standardise this dimension below the lateral pole of the condylar head. The co-ordinates of these locations were noted.

These co-ordinates in (X, Y, Z planes – minimum and maximum) were re-verified before continuing. These new clipbox co-ordinates now identify the maximum linear antero-posterior, superior-inferior and medio-lateral dimensions in which the condylar cartilage was situated in.

Though samples were identified by their animal designation, all attempts were made so that all samples were measured randomly and blinded from day and group influences.

9.8.2 Analytical assessment of the total volume of the condylar head

The orientated dataset ([sample designation]_orient) was then re-imported into VGStudioMax with the new clipbox co-ordinates. No thresholding was done, but the image was smoothed with a Gaussian filter (3x3x3 voxels).

Examining the antero-posterior axial slices, a suitable slice (See Appendix 15.4) was located in which the demarcation of stained condylar cartilage was clearly identified, highlighted by the uptake of the Gadolinium staining medium. The 3D segmentation tool on VGStudioMax, using a gray scale tolerance level of 100, was arbitrarily selected on a section of condylar cartilage. Static mode was used to prevent the software from adaptively changing the tolerance level. The segmentation tool is designed to select similar gray scale values with a tolerance level, and as such, the tolerance level was altered accordingly until the condylar cartilage, periosteum and trabeculae of the bone was selected without the selection of data related outside these defined boundaries (See Appendix 15.4). This tolerance level was noted and the selection area was expanded until the selection area increased (~25-28 um^3 = 4 times expansion in 7um scan samples and 5 times expansion in 5um samples) in all three planes of space to consolidate the selection area. This universally removes as much voids as possible within the condylar head and adjacent ramus before being accepted (See Appendix 15.4). The selection was segmented from the noise and assessed three dimensionally to ensure selection was satisfactory. Any additional noise identified was further segmented until as much of the

noise adjacent to the condylar head was removed without interfering with the structural integrity of the condylar head. All samples were treated identically to allow comparisons between samples.

Once satisfied with the segmentation of the condylar head and associated ramus, the volumetric properties of the whole sample (in voxels) were noted into a Microsoft Excel 2003 Spreadsheet and the sample's datasheet (Table 1). Additionally, 3D images of the sample were also taken for reference and notes made of the segmentation procedure as well as the overall 3D representation of the sample (Figure 17). Anterior volumetric measurements were taken as the difference between posterior and whole total volumes.

9.8.3 Analytical assessment of the total cartilage volume of the condylar head

The specimen was further segmented to select only the cartilage. Again an arbitrary tolerance value of 100 was used when selecting the cartilage and the tolerance level varied until only the cartilage was selected and not the adjacent bone marrow of the ramus. This selection was then expanded (~20-21um^3 = three times in 7um scan samples and four times in 5um scan samples) to universally remove as much voids as possible within the condylar cartilage before being accepted. The cartilage was then segmented and viewed three dimensionally to ensure the selection was satisfactory. All samples were treated identically to allow comparisons between samples.

Once satisfied with the segmentation of the condylar cartilage, the volumetric properties of the whole sample (in voxels) were noted into a Microsoft Excel 2003 Spreadsheet and the

sample's datasheet (Table 1). Additionally, 3D images of the sample were also taken for reference and notes made of the segmentation procedure as well as the overall 3D representation of the sample (Figure 17).

9.8.4 Analytical assessment of the Posterior total volume of the condylar head

The newly identified clipbox measurements were used again to reduce the region of interest of the "orientated" sample to only the posterior portion and the resultant dataset imported into VGStudioMax. The image was smoothed via a Gaussian filter (3x3x3) once to improve the segmentation procedure.

An axial slice was then examined to identify a suitable slice that clearly identified condylar cartilage, highlighted in bright white by its uptake of the Gadolinium staining medium. The segmentation tool, using the "Static" value obtained for the whole condylar head and ramus, an area of condylar cartilage was then selected. The area selected by the segmentation tool was evaluated and the tolerance level altered accordingly until the area selected consisted primarily of the condylar head and associated medullary bone, with minimal noise selected in the adjacent area. The selection was then expanded, as described previously for the total volume, to universally remove as much voids as possible within the condylar head and adjacent ramus and accepted. The selection was segmented from the noise and assessed three dimensionally to ensure selection was satisfactory. Any additional noise identified was further segmented until all, if not most of the noise adjacent to the condylar head was removed without interfering with the structural integrity of the condylar head. All samples were treated identically to allow comparisons between samples.

Once satisfied with the segmentation of the condylar head and associated ramus, the volumetric properties of the whole sample (in voxels) were noted into a Microsoft Excel 2003 Spreadsheet and the sample's datasheet (Table 1). Additionally, 3D images of the sample were also taken for reference and notes made of the segmentation procedure as well as the overall 3D representation of the sample (Figure 17).

9.8.5 Analytical assessment of the posterior cartilage volume of the condylar head

The specimen was further segmented to select only the cartilage. Again an arbitrary tolerance value, previously obtained for the whole cartilage, was used when selecting the cartilage and the tolerance level varied until only the cartilage was selected and not the adjacent bone marrow of the ramus. This selection was then expanded, as discussed previously for cartilage, to universally remove as much voids as possible within the condylar cartilage before being accepted. The cartilage was then segmented and viewed three dimensionally to ensure the selection was satisfactory. All samples were treated identically to allow comparisons between samples. Anterior volumetric measurements were taken as the difference between posterior and whole cartilage volumes.

Once satisfied with the segmentation of the condylar cartilage, the volumetric properties of the whole sample (in voxels) were noted into a Microsoft Excel 2003 Spreadsheet and the sample's datasheet (Table 1). Additionally, 3D images of the sample were also taken for reference and notes made of the segmentation procedure as well as the overall 3D representation of the sample (Figure 17).

A repeated measurement of all volumetric measurements was conducted months later to assess the reproducibility of measurements and method error. These repeated measurements were also averaged with the original measurements for statistical analysis.

9.9 Statistical Analysis

Prior to statistical analysis, the following information was attained and calibrated to metric units (see Appendix 1.1):

(1) Sample name

(2) Class (0, 2, 3 – Control group, Posterior (BWD) displaced mandible group and Anterior (FWD) displaced mandible group respectively)

(3) Day of Sacrifice

(4) Total volume of whole bone and cartilage (cubic millimeter)

(5) Total volume of cartilage (cubic millimeter)

(6) Anterior volume of whole bone and cartilage (cubic millimeter)

(7) Posterior volume of whole bone and cartilage (cubic millimeter)

(8) Anterior volume of cartilage (cubic millimeter)

(9) Posterior volume of cartilage (cubic millimeter)

(10) Maximum antero-posterior (AP) dimension (micrometer)

(11) Maximum supero-inferior (SI) dimension (micrometer)

(12) Maximum medio-lateral (ML) dimension (micrometer)

(13) Half antero-posterior (½AP) dimension (micrometer)

(14) Maximum Cubic dimension (AP x SI x ML – cubic millimeter)

(15) Maximum anterior cubic dimension (½AP x SI x ML –millimeter)

(16) Weight at appliance placement (grams)

(17) Weight at time of appliance removal (grams)

(18) Weight at time of sacrificed (grams)

The raw data was then analyzed by SPSS software (V16.0 SPSS, USA), in consultation with statisticians (P. Petocz, T. Prvan). A univariate analysis of variance (ANOVA) was used to assess the following variables at a confidence level of $p<0.05$. No significant differences were noted between the first and the repeated measurement. Hence averages were taken of both the first and the repeated measurements to allow the following variables to be examined by the statisticians:

9.9.1 Volumetric Measurement Analysis

(1) Total volume (bone/cartilage/periosteum) of the whole condylar head (avTvol)
(2) Posterior total volume (bone/cartilage/periosteum) of the posterior hemisection (avTvolP)
(3) Total cartilage volume (cartilage/periosteum) of the whole condylar head (avTcart)
(4) Posterior cartilage volume (cartilage/periosteum) of the posterior hemisection (avPcart)

These dependent variables were analysed against fixed factors of sacrifice date (Day) and jaw relationship (Class) and the interaction between Day and Class. Due to the notable difference in weight between control and experimental animals at the time of sacrifice, a covariate was used to compensate for possible differences in animals sizes. A covariate is a variable that is suspected of influencing the results, but is not a variable of interest. The maximum cubic dimensions, dictated by the maximum linear (AP, SI, ML) measurements and the weight at sacrifice were examined for their use as a covariate. Tcube was found to be a better covariate of the two and hence was used for compensate for differences between animal sizes.

The data was then analysed further with Pairwise comparisons of the Estimated Marginal Means, using a Bonferroni adjustment for multiple comparisons for both Day and Class Fixed factors.

9.9.2 Method Error Analysis

An intra-individual method error was also conducted with the repeated measurements with an error analysis based on only having at most two measurements for each case. This was conducted with a paired sample T-test that examined the paired differences. A coefficient of variation was also developed for an error analysis that can extend to having more than two measurements for the same variable on each case.

10 Results

All animals survived the experimental period with no premature losses. Daily activity was assessed daily with no obvious signs of distress by any of the animals during the experiment in either control or experimental groups. All animals appear to have been fed sufficient quantities of Nutrigel as daily recordings of feeding identified that over a 24-hour period most, if not all, the Nutrigel was consumed in this period.

Of the 70 right hand hemisections taken for analysis, only 67 samples were successfully measured. Three samples were rejected (GLD004 – Day 0 Controls; GLD009 and GLD010 – Day 7 Controls) as the condylar head was displaced very close to the adjacent glenoid fossa. This meant there was limited separation between these two anatomical structures. Thus, segmentation and analysis of these samples was extremely difficult. Initial analytical measurements suggested that there was a low signal to noise ratio (i.e. excessive imaging noise or irrelevant data) that inhibited accurate measurements to be taken from these samples.

10.1 Imaging Protocol

The protocol developed especially for this experiment allowed image analysis to be predictable and highly reproducible in the majority of samples. As the position of the condyle varies in relationship to its adjacent fossa, care was taken to ensure this position was not altered during the specimen preparation stage. Unfortunately, in some cases, the condyle closely approximated the adjacent cranial vault or overlying zygomatic arch. These images

underwent further segmentation to remove the noise generated by these adjacent structures, while maintaining the condylar head segmentation, improving the signal to noise ratio. This was successful in the majority of cases. However, in a limited number of cases, approximation between the condylar head and the adjacent anatomical structures was so close that it was difficult to remove the excess noise, without affecting the condylar head, or even resulted in the two anatomical structures merging. Samples were reviewed stringently in these cases to remove as much noise as possible, without affecting the segmentation of the condylar head. As mentioned previously, three samples were removed from the study as they were too difficult to analyse.

10.2 Method error and intra-individual reliability

Overall, the sample analysis protocol developed in this study proved to be very reliable as a repeated measurement of the samples was undertaken months after initial measurement. A paired sample T-test was used to assess the differences between original and remeasured variables. The error analysis was based on only having at most two measurements for each case. No significant differences were noted between the original and remeasured values for any of the variables examined (Total volume, Total cartilage volume, Posterior total volume and Posterior cartilage volume). A coefficient of variation was developed for the error analysis that could extend to having more than two measurements for the same variable on each case. The co-efficient of variations ranges from 5.37% to 13.32% (See Appendix 15.7.9). This suggests that this protocol was highly reliable with minimal differences in measurement values with repeated measures.

10.3 Quantitative Analysis

Volumetric measurements of Total volume, Total Cartilage volume, Posterior Total volume and Posterior cartilage volume were analysed using a statistical program (SPSS V16.0, USA). These were assessed in relation to Group (Class) and Day using a Univariate Analysis of Variance. Since the error analysis showed no significant differences between any volumetric measurements, the original and remeasured values were statistically analysed as average volumes of the four dependent volumetric measurements. Due to the significant weight differences between samples, the maximum linear measurements (antero-posterior, supero-inferior and medio-lateral) were used to develop a cubic volumetric measurement (Total cube – Tcube). This was used as a covariant to compensate for differences in animal sizes, and proved more reliable than weights at sacrifice. Statistical data have been tabulated in tables (Table 4, Table 5 and Table 6).

10.3.1 Total volume

Total volume (AvTvol) is the volumetric measurement of the whole condylar head encompassing bone, cartilage and periosteum. Independent of group (Class), over the duration of the experiment AvTvol reduced significantly ($p<0.001$). Further examining differences between time point, total volume ($p<0.002$) was significantly reduced between the start of the experiment and every other time point (Days 7, 21, 28; $p<0.002$). However, at each time point, there were no differences between any of the three groups. Hence the duration of the experiment had an effect on the AvTvol.

Examining the changes occurring within each group, Controls demonstrated a significant reduction in volume over the experimental period ($p<0.001$), particularly between Day 0 and Days 21 ($p<0.005$) and 28 ($p<0.001$). However BWD and FWD groups demonstrated a non-significant reduction in total volume over the same time period. Therefore, in normal growth, there is a reduction in AvTvol, however the presence of appliance had no difference on this volume over the duration of the experiment.

Examining the changes occurring at each time point, there was no significant difference in AvTvol between any of the three Classesroups at Days 0 and 28. But at Day 21, a significant difference was noted between groups ($p<0.005$), with Controls being significantly larger than BWD animals ($p<0.001$).

10.3.2 Total Cartilage Volume

Total cartilage volume (AvTcart) is the volumetric measurement of the cartilage and periosteum of the whole condylar head. Similar to AvTvol, AvTcart demonstrated a significant reduction in volume over the duration of the experiment independent of group ($p<0.001$). Further analysis demonstrated that this significant reduction in volume were also noted between the start of the experiment and every other time point ($p<0.001$). However unlike AvTvol, there were significant differences in AvTcart in relation to group, independent of time intervals ($p<0.005$). Further analysis demonstrated that the differences were seen between the FWD group, which had significantly larger mean volumes than the Controls ($p<0.001$). Appliances appear to induce a significantly ($p<0.005$) larger volume of cartilage over the duration of the experiment. FWD groups demonstrated a larger mean volume than Controls ($p<0.001$).

Examining the differences between groups over the duration of the experiment, Controls demonstrated a significant difference in AvTcart ($p<0.005$). This difference were noted between Days 0 to 21 ($p<0.005$) and 28 ($p<0.05$) with a reduction in AvTcart over time. Alike the AvTvol, FWD and BWD demonstrated a non-significant reduction over the same time period.

Similar changes seen in AvTvol were noted for AvTcart at each time point. There was no significant difference between Classes at Day 7 and 28. However at Day 21, there was a significant difference ($p<0.01$) between groups with FWD groups having significantly larger mean volumes than Controls ($p<0.01$).

10.3.3 Total Posterior Volume

Total posterior volume (AvTvolP) is the volumetric measurement of the bone, cartilage and periosteum of the posterior hemisection of the condylar head. Similar to the previous volumetric measures, there is a significant reduction in AvTvolP over the duration of the experiment ($p<0.05$), particularly between Day 0 and Days 21 and 28 ($p<0.05$), independent of groups. Similar to AvTvol, there were no effects of groups on AvTvolP over the duration of the experiment.

Further analysis of AvTvolP changes over the duration of the experiment in each group, no significant differences were noted in any of the groups at any time intervals.

When examining the different time intervals, there was no significant difference in AvTvolP at Day 7 or 28 with any of the groups. But at Day 21, a significant difference between group

was noted ($p<0.05$) with BWD animals demonstrating a larger volume than Controls ($p<0.005$) and FWD ($p<0.005$).

10.3.4 Posterior Cartilage Volume

Posterior Cartilage volume (AvPcart) is the volumetric measurement of cartilage and periosteum in the posterior hemisection of the condylar head. A general decline was noted in the posterior cartilage volume ($p<0.05$) with a significant difference noted between Day 0 and 21 ($p<0.05$) independent of group. Analysis of the differences between each group over the whole experimental duration, demonstrated a significant difference between Classes ($p<0.05$). This difference was noted with FWD groups having significantly larger mean AvPcart volumes than Controls ($p<0.001$).

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Posterior cartilage significantly reduced over the experimental period in Controls ($p<0.05$), particularly between Day 0 and 21 but there was no significant differences seen in posterior cartilage volumes in both BWD and FWD groups over the duration of the experiment.

No significant differences were noted between Class groups at Day 0 and 28. However there was a significant difference in AvPcart with BWD ($p<0.05$) and FWD ($p<0.05$) being larger than Controls at Day 21.

10.4 Qualitative Analysis

Besides quantitatively analysis, images were taken to allow gross morphological changes to be assessed qualitatively. A visual assessment of the samples was carried to identify changes

in the shape of the condylar head in both Control and experimental groups. This was then compared over the four time points as well between experimental and control groups.

10.4.1 Volumetric Measurements

10.4.1.1 Control groups (Class 0)

Gross assessment of the condyle at Day 0 illustrated that the condylar head of the Sprague Dawley rat varies typically from a symmetrical ovoid shape (
Figure 19) to a tear drop shape (posteriorly weighted -
Figure 20), with an increased propensity of bone and cartilage to be present in the posterior hemisection when viewed superiorly. The medial and lateral poles of the condylar head were also clearly visible and could be identified when the sample was viewed from the lateral direction. This provided the demarcation used to allow for the orientation of the samples as described previously.

This distinctively separates the condylar head from the ascending ramus inferior to it. Finally another distinctive feature of the condylar head is the posterior border when viewed laterally. This varied from being a round convex surface (Figure 21) extending superior to inferior but may also appear a more pointed surface (Figure 22).

Over time, the general ovoid shape of the condylar head was maintained, with a tendency for a more centrally weighted shape, whereby the majority of the bone and cartilage was present

in the middle of the sample, rather than the posterior end as illustrated at Day 0. This becomes increasingly the case from Day 7 (Figure 23), 21 (

Figure 25), and 28 (

Figure 27). The general convexity of the posterior border of the condylar head and adjacent ascending ramus was maintained as well as the well defined lateral and medial poles of the condylar head (

Figure 24,

Figure 26,

Figure 28).

10.4.1.2 Experimental groups

10.4.1.2.1 Posterior Displacement of the Mandible (Class 2):

10.4.1.2.2 Day 7 (BWD Day 7 – Class 2):

Animals in which the mandible was maintained in a posteriorly directed position (Class 2) for only a week, no obvious differences were found in the overall shape of the condylar head or in any of the other characteristic features (

Figure 29,

Figure 30) compared to Day 0 and 7 Controls. There was a tendency for the condyle to be more centrally weighted than posterior weighted, which was consistent with controls over the experimental period.

1.1.1.1 Day 21 (BWD Day 21 – Class 2):

After 21 days of appliance placement, the overall shape of the condylar head was maintained when compared to control and BWD Day7 groups. Again, consistent with Control groups over time, the shape of the condyle tends to be increasingly centrally weighted, giving the condyle a more rectangular shape (

Figure 31,

Figure 33). The superior surface of the condylar head however appeared to be slightly flattened with time and the posterior boundary of the condylar head, when viewed superiorly, also appeared flattened. Remodelling was evident on the posterior border of the condylar head (

Figure 32,

Figure 34).

When compared to Control groups (Day 0, 7 and 21), the BWD Day 21 groups exhibited a more flattened superior surface and demonstrate evidence of posterior remodelling, typically as either the development of a noticeable notch on the posterior border or flattening of this curvature compared to the general convexity seen in control groups. When compared to the FWD Day 21, more obvious notching on the posterior border (

Figure 35,

Figure 36,

Figure 39) or significant remodelling (

Figure 37,

Figure 38) was noted in FWD Day 21 samples.

10.4.1.2.3 Day 28 (BWD Day 28 – Class 2):

After removal of the appliance on Day 21, animals sacrificed at Day 28 also demonstrated an overall condylar head shape similar to controls, though there was a propensity for the condylar shape to be more centrally weighted (
Figure 40). These samples also demonstrate flattening of the superior surface as well as the posterior border of the condylar head (
Figure 41,
Figure 42).

BWD Day 28 samples demonstrated a flatter superior surface compared to control groups (Day 0, 7 and 21) as well as BWD Day 7 and BWD Day 21 groups (

Figure 42). However a similar flattening of the superior surface was noted on both BWD Day 28 and Day 28 Control groups. The general shape of the condyle was maintained in BWD Day 28 samples relative to Controls (Day 0, 7, 21, and 28), however the posterior border of the condylar head was noted to be more flattened or exhibited remodelling in the BWD Day 28 Samples. When compared to FWD Day 28 samples (

Figure 43), the FWD Day 28 samples exhibited a more irregular and mottled shape, with similar superior surface flattening. The posterior border of the condylar head was more notched or flattened in FWD Day 28 samples (

Figure 44).

10.4.1.3 Anterior displacement of the mandible (Class 3):

10.4.1.3.1 Day 7 (FWD Day 7 – Class 3):

Animals in which the mandible was maintained in an anteriorly directed position (FWD), there was also no difference in its overall shape when compared to Day 0 and 7 Controls (
Figure 19,
Figure 45). There was also a tendency for the condyle to be more centrally weighted, consisted with controls over time and BWD Day 7 experimental group. Due to the change in the conformational shape of the condyle, the overall shape of the condylar head tends towards being a square/rectangular shape. However, when compared to the BWD Day 7 experimental group, the superior surface of the condylar head appeared to be slightly flattened. These samples also exhibited a tendency for the posterior border to be flatter than the anticipated convex shape or even exhibit a more irregular shape, suggestive of remodelling (
Figure 46,
Figure 47, Figure 49). Remodelling was also noticed when viewed superiorly, with the normal round posterior surface of the condylar cartilage, appearing more irregular (
Figure 45).

10.4.1.3.2 Day 21 (FWD Day 21 – Class 3):

FWD Day 21 samples exhibited slight flattening of the superior surface with a condylar shape which appeared more square/rectangular relative to Day 0, 7 and 21 Controls (

Figure 25,

Figure 37), as a result of the shift from a posteriorly weighted tear shape to a more centrally weighted shape. There also appears to be a reduction in the medio-lateral width in these samples. Distinct notching or flattening of the posterior border was noted in these samples (

Figure 35) with remodelling of the condylar head evident in some samples (

Figure 38) compared to Day 0 and Day 7 Control groups.

10.4.1.3.3 Day 28 (FWD Day 28 – Class 3)

Overall the gross condylar shape demonstrated significant remodelling with the condylar head appearing mottled and irregular in shape (

Figure 43). The overall shape of the condyle appeared rectangular in shape rather than tear drop shaped. Posterior notching was noted in a large number of samples (

Figure 44,

Figure 46,

Figure 47). Compared to Controls (Day 0, 7, 21, 28), FWD Day 28 samples typically were more rectangular shape than tear drop with the superior surface being flatter. FWD Day 28 samples also demonstrated remodelling, posterior notching and loss of the typical ovoid shape.

10.4.1.4 Weight of Animals:

Weights were not taken prior to the commencement of the experiment, but assuming that all rodents were received from a scientific laboratory designed to specifically breed these rats, it was assumed that all rats weighed approximately the same at the commencement of the experiment. All animals were weighed at time of sacrifice, but experimental were also weighed at the time of appliance placement in all experimental groups, and in Day 28 experimental groups, at time of appliance removal.

Examining the weights at sacrifice over the duration of the experiment, control groups demonstrated a non significant reduction in weights. In contrast, both BWD ($p<0.001$) and FWD ($p<0.001$) demonstrated significant reduction in weight from Day 0 to 28 cross-sectionally. Significant differences were noted in comparisons between Day 0 and Days 7, 21, and 28 ($p<0.05$).

Assesssing the differences in weight at each time point, there were significant differences between Appliance groups and Controls. At Day 7 and Day 28, FWD animals were significantly smaller than Controls ($p<0.01$). At Day 21, both BWD ($p<0.05$) and FWD ($p<0.01$) were significantly smaller than Controls.

11 Discussion

11.1.1 Summary of Quantitative results

In summary, all volumetric measurements demonstrated a general reduction in volume over the duration of the experiment, with significant difference noted between the start of the experiment (Day 0) and every other time point (Day7, 21 and 28).

Total and Posterior Cartilage volumes also have differences between groups, independent of time intervals, with appliances groups appearing to have significantly larger volumes of cartilage throughout the duration of the experiment relative to Controls.

Further in-depth analysis identifies subtle changes between different groups and Day intervals. Experimental groups demonstrate a non-significant reduction in four volumetric measurements over the experimental period unlike Controls.

Significant changes tend to occur at Day 21. Controls had significantly larger total volumes than BWD. Conversely, BWD had significantly larger posterior volumes than Controls and FWD. With both Cartilage volumes being significantly greater than controls in appliance (BWD and FWD) groups.

11.1.2 Summary of Qualitative results

Conformational changes in the overall shape of the condyle were noted in appliance groups as seen from a superior and lateral view. Although controls demonstrate a general shape change over time, the condyle appeared clearly defined and consistent between samples. Appliance (BWD and FWD) groups demonstrating varying changes in relation to the shape of the condyle. BWD condyles typically demonstrated similar changes as controls over time, though there were evidence of remodelling changes occurring morphologically. These changes were seen as a flattening of the superior surface, an irregular condylar outline and the development of a notch along the posterior border of the ascending ramus. FWD condyles exhibited more characteristic remodelling changes, often appearing very irregular. Compared to the changes demonstrated in BWD condyles, remodelling was markedly more dramatic. The condyles often appear in stark contrast to corresponding control condyles. Hence significant morphological changes are detected with the placement of the appliance and the duration of the experiment.

11.1.3 Conclusions of this investigation

Qualitatively and quantitatively, there were marked differences in all groups over the 28-day period. Controls exhibited a general change in overall shape but also demonstrated a reduction in total volume throughout the experiment for all volumetric parameters.

Though experimental groups demonstrated a non-significant reduction of all volumetric parameters over the duration of the experiment, marked differences were seen. Total and posterior cartilage volumes appear to have increased over the duration of the experiment with the placement of appliances. Morphologically, these experimental groups also exhibited marked changes in shape and overall structure. These were visualised at Days 21 and 28, which correspond with the statistically significant differences not detected quantitatively. With a general reduction in Total volume over time, controls demonstrated a significantly larger total volume than BWD groups at Day 21. However, there appears to be differences in the distribution of this reduction in volume, with BWD groups demonstrating a larger posterior total volume than Controls.

11.2 Condylar cartilage changes

Currently there is no literature describing the changes in condylar cartilage volumes as a result of orthodontic intervention. The literature on condylar cartilage changes as a function of appliance placement has been restricted to cartilage thickness measured from histological sections.

Aside from the obviously marked anatomical differences between the TMJs of rodents and man, there have been differing reports on the adaptive response of the rodent joints after treatment. Tsolakis and Spyropolous [113] have suggested that the differences between such observations were the result of the anatomical characteristics of the dental arch and mandible, as well as the design and fit of the appliances. [56]

11.2.1 Controls

The literature on the effects of functional appliances on cartilage thickness demonstrates a variation in thickness along the circumference in controls. The articular tissue varied in thickness, being thickest in the posterior region, and becoming progressive less thick through the posterosuperior and superior regions. [90] McNamara et al [90] determined that the cartilage in the posterosuperior region was approximately one and a half times thicker than the posterior region and almost twice as thick in the superior region. With normal growth, the thickness of cartilage layer decreased two dimensionally. Although this investigation examined the changes three dimensionally, this investigation also demonstrated a reduction in the total cartilage volume with growth.

11.2.2 Appliance placement

When animals were subjected to functional appliances, the articular layer was found to be slightly thicker than controls, but with no apparent relationship between variation in thickness and treatment or duration of treatment. [90] Studies of muscular dystrophic mice, demonstrated a thicker condylar cartilage [114] and a differing shape of the condyle. [114] Similarly, this investigation found that total cartilage volumes were larger than Controls. This is in contrast to Ghafari and Degroote [64] who measured the overall thickness of the condylar cartilage histologically. They found that the condylar cartilage thickness was less in the animals treated with bite jumping appliances than in the Controls, although the difference was not statistically significant.

However, contrary to McNamara et al [90] and Ghafari and Degroote, [64] this investigation demonstrated that appliance placement resulted in a non-significant change in cartilage volume in comparison to the significant reduction in cartilage volume seen in controls. Anterior displacement (FWD) demonstrated a significantly larger total cartilage volume than Controls.

McNamara et al [90] found that the principal area of adaptation was along the posterior border of the condylar cartilage. Adaptations were also noted on the posterior border of the ramus. This is consistent with the qualitative analysis of this current investigation, which demonstrated that changes were evident in the posterior boundary of the condylar head and the posterior border of the ramus. Appliance groups resulted in a significantly larger volume than controls at Day 21, consistent with McNamara et al. [90]

Though the literature suggests that compressive loading on the mandibular condyle reduces the thickness of cartilage *in vitro* [54] and *in vivo*, [115] this current investigation demonstrated that compressive loads had a non-significant difference in Total cartilage volume compared to Controls or Anterior displacement of the mandible.

McNamara et al reported that the superior condylar region was not significantly affected by the functional protrusion appliance. [90] However Ghafari and Degroote [64] stated that a decrease in force levels through forward and downward shift of the mandible, with subsequent reduction of biting forces and dietary alteration, could account for the smaller condylar cartilage thickness and area in the displaced mandibles observed. [64] The results of this study concur with Ghafari and Degroote, [64] as morphologically, appliance placement has an effect on the superior surface of the condyle. Additionally, all volumetric measurements

reduced over the experimental period with smaller condyles at the end of the experiment, suggestive of load reduction via diet. Though the reductions in appliance groups were not significant between Days 7 to 28, they were significantly different to Control during this same period.

Remodelling as a result of appliance placement have been suggested by a number of researchers. Cholasueksa et al [72] suggested that the cartilage width decreased as a result of changes in the microenvironment of the condylar cartilage. They found the abrupt change in the condylar cartilage response was due to a change in loading, resulting in a significant decrease in cartilage width, as chondroprogenitor cells for the growth cartilage were destroyed. [72] Interestingly they found that condylar cartilage, over time, was able to recover and eventually exhibit increased thickness of the cartilage layer compared to control, but cartilage degeneration still persisted up to 8 weeks after appliance placement. [72] This may explain why no significant differences were noted after appliances were removed at Day 21 in this current investigation.

Asano et al indicated that growth retardation and enhancement were the likely results of adapting to the orthopaedic force and that the mandible remodelled its form and structure to accommodate the force and altered environment. [73] These suggestions concur with Teramoto et al, [56] who concluded that cartilage volume tends to decrease by the loss of physiological force (e.g., joint movement, masticatory force), and by static compressive force, whereas it tends to increase with adequate force, including intermittent force.

Due to the conflicting evidence, differences observed in rat and monkey studies may also be attributed to the relative magnitude of mandibular displacement due to appliance design,

species differences in the adaptive potential of the condylar cartilage, [116] and/or the different genetic growth programs in rats (vertical condylar growth direction) and monkeys (upward and backward condylar growth direction). [53]

11.2.3 Linear measurements

This study demonstrated an increase in the anteroposterior length of the condylar head over the 28-day period, independent of group. McNamara et al [64] also found that the overall length of the condyle did not differ between experimental and control groups. They suggested that the rate of bone resorption anteriorly was higher that would have normally occurred without experimental intervention. This may be a possible explanation in this study, as cartilage volumes increased, though total volume of the whole condylar head was maintained during the experimental period. Yamada et al [50] also found thicker cartilage when the condyle was not loaded with a hard diet. Usually thicker cartilage has been related to increased condylar growth. This would imply that there would be increased condylar growth and a larger condylar length. But Yamada et al [50] found the converse occurred and postulated that the increased thickness may be due to differing composition of the cartilage layers with differing rates of differentiation and maturation of mesenchymal cells into chondrocytes, reflected by the various loading patterns of the cartilage. Similarly, this was seen in the posterior cartilage volume of FWD groups in this investigation.

11.2.4 Effects of differential loading of the condyle

There is sufficient literature to support the possibility that the effects of differing loads applied to the condylar cartilage, alters its response.

The use of asymmetric occlusion by means of unilateral incisor trimming, have been used to assess the effects of differential loading and unloading of TM joints. Despite unilateral incisor trimming, there were no significant differences observed between the different groups of cartilage sections. However, it was noted that the cartilage was slightly thinner on the untrimmed (unloaded TMJ) side compared to control and the trimmed (load applied TMJ) side, which showed similar cartilage thickness. [47] After 7 days of incisor disocclusion, this thinner cartilage thickness was still detected on the untrimmed side. It has been postulated that this may be a sign of an initial change in thickness which might show up with a longer time interval [56] as demonstrated by other studies [117] In contrast, FWD groups, in which the condyle has been distracted from its articulation, hence is unloaded, demonstrated larger cartilage volumes than Controls at Day 21. BWD groups also demonstrated an increase in cartilage volume relative to Controls, even though they were displaced more posteriorly, increasing the load applied. Furthermore, when examining the changes in posterior volumes, BWD groups in which the condyle were subjected to more loads, demonstrated an increase in cartilage volumes compared to the Controls.

When examining the differences in condylar cartilage thickness due to altered loading of the condyle via different dietary consistency, it was found that the condylar cartilage was thin in its anterior part, before gradually thickening in the posterior part and this was noted in all

portions (lateral, central and medial). In rats fed a soft diet, the condylar cartilage was thinner in the anterior part of the condyle compared to rats fed a hard diet. Conversely, the condylar cartilage was thicker in the posterior part of the group in the soft diet fed group [50] hence topographic changes in the condylar cartilage thickness are noted with a low masticatory function. This is consistent with previous findings in which a reduced masticatory function influenced the thickness of the condylar cartilage. [53,59,60] This investigation also found that there was an increase volume in total and posterior cartilage volumes in appliance groups.

There was also an effect on bone volume, with reduction in bone volume in conjunction with reduction in masticatory function. [49] Yamada suggested that, normal mechanical loading of with normal diets, seemed important for periosteal bone cell and cartilage cell activity in the growing mandible. [49] Thus functional loading of the condyle is an important process of increasing the bone mass in different parts of the mandible to better withstand higher functional demands. [50] This is a physiologic process observed in different bones, both in animals and in humans. On the contrary, cartilage volumes were larger in FWD groups than controls for posterior cartilage volume, when the condyle is unloaded from its articulation. Generally, there was a decrease in volume in all groups over the duration of the experiment; however BWD exhibited significantly greater volumes in the posterior. This is suggestive of a response to increased functional demands.

11.3 Review of Study Design

The study design of this investigation closely mimicked that of previous studies [87,118-121] involving bite jumping appliances and Sprague Dawley Rats. However, due to issue with animal anatomy and differing patterns of attrition, the maintenance of a bite jumping posture

was not always possible. Thus the jaw posture of experimental groups varied from a mandibular anterior displacement (FWD – Class 3) or a mandibular posterior displacement (BWD – Class 2). Inadvertently, this reduced our small sample size significantly.

All animals appeared healthy and active throughout the experimentation, without any premature loss of any animals due to misadventure. Strict protocols were in placed to monitor their welfare throughout the experimentation.

11.3.1 Animal Model – Sexual maturation

Growth potential is essential when applying orthopaedic interventions to achieve clinical outcomes. It is noted that sexual maturation is a good indicator of pubertal growth peak, where the growth rate is highest. [122] Sexual maturation is one of many developmental landmarks used to assess postnatal physical development in rodents. [110] Because these maturational endpoints do not occur until after weaning, the reported values are usually based on observations for one randomly selected rat/sex/litter. [110] However, there are issues regarding determination of sexual maturation of Sprague Dawley rats. Lewis et al [110] compared the sexual maturation data from various types of littering studies using the International Gold Standard Sprague-Dawley rats with both historical observations and published values from other laboratories. They found that there were statistically significant and biologically important differences. They concluded that one of the most important confounding factors identified for sexual maturation data was the absence of a standard endpoint criterion across testing facilities with inter-laboratory differences in published sexual maturation values. [110] Hence, the variability of sexual maturation data from

commercial laboratories may have resulted in the use of animals that were not at the peak of their sexual maturation.

Innumerable theories have been postulated to explain the onset of sexual maturation. One prevailing theory suggests that body weight or percent body fat correlates with the initiation of puberty, especially in female rats. [110] This theory was supported by the fact that human females require a minimal level of adipose tissue to achieve menarche (approximately 17% of total body tissues) and to maintain reproductive competence (approximately 22% of total body tissues). [110] It is well established for female humans and rodents that prepubertal body weight reductions can result in significant delays in the onset of puberty and loss of fertility. [110]

Considering that there is a lack of literature discussing the effect of appliances on weight gain,[64] there are few previous studies that have mentioned the ability of animals, subjected to the placement of an appliances, to maintain weight or function after appliance placement. Ghafari and Degroote [64] discussed the effects of oral appliances and the weight of animals during their experiment. They found that at the end of the experiment, the experimental animals had gained less weight than the control animals, demonstrating that the normal function of the experimental animals was impaired by the oral device. [64] Tonge et al [52] found that their animals had difficulty adjusting to the anterior bite plane, which was reflected by the failure of the experimental animals in the 7-day experiment to gain weight. After one month of appliance wear, the experimental animals did record an increase, but the mean weight gain was only half that of the controls. [52] This current investigation also demonstrated that appliances had a significant effect on weight over the duration of the experiment. Significant weight reduction at sacrifice was noted cross-sectionally in both appliance groups.

But in contrast to animals of these previous studies, [52,64] the animals in this study had their diet tightly controlled. Ghafari and Degroote [64] commented that the loss of weight could have been attributed to the effects of repeated general anaesthesias conducted on animals who had appliances removed daily, or the inefficiency of a fixed anterior bite plate.

However, more substantially was the weights of all animals compared to their anticipated weights at the time of sacrifice. Standard Sprague Dawley Rat charts supplied by commercial rat breeding laboratories, calculate the age of the animals relative to their weight. These standard weight charts demonstrate a rapid increase in weight of 30g (5 weeks) to 100-150g (9 weeks). [123] Hence, five week old rats were chosen for their pubertal growth spurt during this time, in accordance with commercial rat breeding laboratories and other rat studies conducted to assess orthopaedic manipulation of the mandibular condyle. [87,118-121] However this investigation demonstrated that no animal gained weight as anticipated, rather maintaining their starting weights or demonstrating a significant decline. Control groups exhibited a non-significant decrease in weight over the experimental period, however definitely did not exhibit the anticipated weight attained at 9 weeks (100-124grams [123]). The experimental animals demonstrated a statistically significant loss of weight by the end of the experiment, with animals weighting between 35-40grams at 9 weeks. Though no pre-operative weights were taken, it was assumed that all animals started at the same weight at Day 0. However, due to the acclimatisation period of 2 weeks prior to the commencement of the experiment, differences in weight may have already occurred. The weights of baseline controls were on average approximately 45 grams, this weight was already significantly less those suggested by standard growth charts by specified by commercial animal laboratories [123,124] which designated females weighing under 50grams as being 18-21days of age (3-4 weeks of age). Hence our experimental animals did not meet the anticipated weight at the

time of commencement. To allow these animals to be compared to animals receiving supplementation, [109] animals were fed a restricted diet of high caloric energy Nutrigel. This has been given to animals previously during recovery of surgery [112] and the feeding regime was similar to that described by Beren et al [125]. This loss in weight may be the result of a controlled diet given to them.

11.3.2 Diet

Though Nutrigel is a high caloric energy supplement, it has not been described as a meal replacement in animals, rather to aid animals in recovery. [112] A number of factors may have contributed to the loss of weight, both at the start of the experiment and during the experiment. It has been recommended that these rats should be fed a commercial rat or rodent diet and water *ad libitum*. These diets are nutritionally complete and do not require supplementation [123]. The daily food intake is approximately to be 5g/100g BW/day with water intake approximately 10-12ml/100g BW/day. The animals in this study were fed 4ml/day of High Energy Nutrigel (Troy Laboratories, Wetherill Park, Australia), which was believed to be sufficient to sustain their health, however there was no data regarding to its use as a replacement to commercial rat or rodent diet, nor its effect on weight and growth of Sprague Dawley rats. Nutrigel was given to the rats in this experiment to provide a vehicle in which medication (glucosamine and chondroitin sulphate) could be administered to the animals in a concurrent study. [109]

Besides the quality and quantity of food given to the animals possibly confounding results, housing of animals may have also confounded the results. The animals were received on two batches over two days. Thirty five animals were divided into control and experimental groups

randomly, resulting in cages housing of four animals in the majority of cases, but cages of three animals in two cages. Individual caging of animals were not financially feasible, but may have provided a way to ensure that a consistent amount of food was taken by each animal. For cages of four animals, 16ml of Nutrigel was given, and in cages of three animals, 12ml of Nutrigel was given. It was assumed that all animals were consuming the same amount of Nutrigel, but it was not feasible to ensure that consumption was equal among all animals within a cage. Food consumption was noted and added to if sufficient was remaining after a 24 hour period. Notably, in the majority of cases, consumption of Nutrigel was completed over a 24 hour period by most cages, but understandably, when appliances were placed, consumption was reduced slightly post-operatively.

Thus the restriction of food intake may have result in the lack of growth of the animals, with the possibility of limiting dietary intake to that required for maintenance nutrition or even sufficiently lower than that. This may not have been sufficient to promote growth or even maturation and consequently may possibly influence our results. For orthopaedic correction to occur favourably, timing of intervention is critical to allow manipulation of growth during its highest growth velocity. [122] As clinical studies on humans have demonstrated, the effects of functional appliances placed in prepubertal children have limited enhancing effects on the growth of the mandible. Resultantly, this may be the case for these animals as well.

Overfeeding by *ad libitum* food consumption is the most significant, uncontrolled variable affecting the outcome of the rodent experiments. *Ad libitum* food consumption adversely affects every physiological process and anatomical structure and affects 2-year survival in Sprague Dawley rats. [126] The restriction of energy intake seems to be the main process retarding aging and improving the longevity of Sprague Dawley rats. [126] Additionally, some

nutritional deficiencies results in changes in the condylar cartilage response. Ascorbic acid deficiency and vitamin D deficiency have been shown to lead to increased cartilage thickness and disturbed erosive activities. [30]

Noticeable weight loss was evident in all groups from the start of the experimentation. Studies have shown that when animals have their diets restricted body mass is rapidly reduced within six weeks in proportion to the reduction of energy intake. [126] It is speculated that diet restricted rats apparently reset their energy utilisation mechanisms to be able to use glucose and insulin more efficiently providing an anti-aging effect [126]. It has been strongly advocated that marked to severe dietary restriction (40-50% of *ad libitum* energy intake) be avoided as a dietary control method. The *ad libitum* consumption of rats ranged from 21.7-32.3g or 264-418kj (63-100kcal) per rat per day. [127]

Future investigations are required to assess the daily nutritional requirements of rats, and compare this with diets replaced by high caloric energy substitutes such as Nutrigel. Furthermore, a nutritionally balanced diet, similar to conventional rat chow, needs to be formulated to provide animals with sufficient fibre and other nutrients to encourage the natural weight gain of rats. If medication administration is not required, conventional rat chow available *ad libitum* would be recommended. Dietary restrictions are not required as suggested by some studies. [126] The short duration of these experiment requires animals to mature as rapidly as possible, rather than the prevent premature organ failure due to over nutrition, found in longitudinal studies of rats used to assess pharmaceutical agents. [126]

Hence the effects of diet restriction, though intended to be a method of controlling medicinal administration, [109] may have inadvertently affected the physiological processes of all rats involved in the study.

Though nutrition was supplemented by a high caloric diet, this may not have been sufficient to promote growth beyond the expected maintenance energy requirements of the animal. Given that *ad libitum* feeding may affect the longevity of animals, studies investigating the growth of the mandible are of relatively short duration. Hence *ad libitum* feeding would be conducive to any future study as it would promote an acceleration of the aging process. [126] Otherwise future investigations should eliminate the use of nutrition replacement, or examined a suitable vehicle to deliver medicinal drugs to rodents.

11.3.3 Appliance placement

The appliance used was similar to previous studies [52,64] in which a lower anterior bite plane was placed to change the pattern of jaw closure. The use of readily available dental materials provided an easy method of placing this plane in all animals in a direct and relatively reproducible fashion, in regards to sizing of the bite plane. However, the effect of the bite planes was not consistent in all animals, though every effort was made to place them in a forward posturing position.

Tsolakis and Spyropoulos [113] found that rats have a wide range of closure patterns that may result in the animal being able to postured forward very far forward, as well as very far backwards. This may have been a confounding factor in our results. Efforts were made to ensure the bites were in a forward direction at the time of surgery and were seen upon

arousal. However, it was difficult to monitor this on a day to day basis thus they were assessed at time of sacrifice. Also, there was evidence that some animals were able to grind their bite planes sufficiently to cause a reversion of the occlusal relationship. In a few animals, high attrition of the upper incisors was seen which was either symmetrical or asymmetrical with fracturing of the incisors.

Other methods have been used to maintain an anterior positioning of the mandible but they are often labour intensive, [64] or require regular monitoring and intervention during the procedure. This is often difficult in animals as they become increasingly resistant to frequent anaesthesia or manipulation. Nevertheless, this method, though requiring alterations to ensure its reproducibility, seems feasible as an alternative to allow orthopaedic intervention.

Another possible issue that may affect the weight of the animals is the cytotoxicity of the dental adhesive and bonding materials used in this experiment. The material data sheet for dental composite and SEP notes it may cause gastrointestinal irritation. This product causes soft tissue chemical burns when in contact with soft tissues. Since SEP was used liberally to help adapt the composite resins to the incisors and paediatric crown formers, it may be possible that SEP may have effects on the soft tissues of the rats, including their digestive system. Further investigations are required to assess the cytotoxic effects on SEP on rats.

11.4 Review of Imaging protocol and measurement

MicroCT has been shown to be effective in the imaging of ex vivo inspection of small bones of small animals. [128] However, the imaging of cartilage has always been difficult in MicroCT

examinations due to their inherently low contrast.[100] With the introduction of gadolinium staining media, this inherent disadvantage of microCT has been quickly overcome.[14,100]

11.4.1 Scanning methodology

Sriram et al[107] demonstrated the ability to use bone staining medium (osmium tetroxide) to improve the visualisation of cartilage with MicroCT. Currently, this study is the first study to look at extensive changes in condylar cartilage three-dimensionally using a gadolinium-based staining medium in conjunction MicroCT x-ray machine. This has enabled the analysis of the changes of the whole condylar cartilage to be assessed in a non-destructive fashion. Gross morphological appearance due to growth and orthopaedic intervention can now be identified, and quantitative measurements taken reproducibly.

MicroCT involves the rotation of a sample while a polychromatic beam is passed through it at specified increments. The rotations used were 0.39 degrees with approximately 923 rotational steps required to image an individual sample. The disadvantage of this MicroCT process is that the rotation steps may cause image malalignment due to the vibration of the specimen platform, as it rotates about its axis. Usually, specimens placed in a MicroCT scanner are often regular shaped or are able to be kept within a specimen container that is sufficiently rigid to minimise rotation vibration. The hemisection of a Sprague Dawley Rat skull is irregularly shaped, requiring the staging platform to be custom fabricated. The staging platform was constructed out of Styrofoam in which the specimen was securely attached by electrical tape. However, its high centre of gravity and the insufficient rigidity of the Styrofoam meant that, as it rotated about its axis, the vibration was sufficient to cause malalignment between rotations. This malalignment was reduced by the alignment tool in the

MicroCT specific software (NRECON, SkyScan, Belgium), by averaging the frames. However, in some cases, software realignment proved difficult to correct for the rotational vibration. Future investigations should investigate the fabrication of a specially designed staging platform made out of rigid materials and with a lower centre of gravity, to aid in minimising this malalignment issue.

Prior to commencement of this experiment, a pilot study was undertaken to determine the suitable staining time and scan resolution required to allow sufficient analysis of the data. Pilot samples were stained in 200mM $GdCl_3$ for 24hours, 3 days, 5-days and 6-days at resolutions of 5um and 7um for each of these time frames. Each scan was then assessed for the ability to segment cartilage from the bone. It was found that 6-days were sufficient to enable replicable segmentation of the cartilage. Furthermore, resolutions were assessed for suitability for analysis. Though 5um provided high resolution, as all slices needed to be rotated to a standard orientation, 5um scans proved limited in its ability to allow a sufficient degree of freedom for structural analysis (Appendix 17.5). Hence, 7um scans provided a suitable compromise between resolution and freedom in rotation of the sample via the specially designed software (RotateScanline).

The segmentation method was shown to be a reproducible method providing a consistent method error (Table 3) and a low co-efficient of variance for multiple measurements (5-13%). Generally this was a simple method, however in some cases; there was sufficient irrelevant noise present in the samples to make segmentation difficult. Noise was predominantly the result of the close proximity of the condylar head to the overlying zygomatic ridge. Thus the definition of the condylar head was not always simple, requiring noise to be separated as much as possible before measurements were taken.

11.4.2 Staining methodology

Reviewing the imaging protocol, we have validated the ability of gadolinium chloride to improve the visualisation of cartilage. [100] The rationale for this staining method was the high affinity of gadolinium to proteoglycans. [14,100] However proteoglycans maybe present in a number of tissues. It was assumed that periosteum consisted of a thin layer which was uniform in thickness over the whole sample. Thus volumetric measurements of cartilage were not solely cartilage only, but were also inclusive of periosteum.

The amount of gadolinium chloride staining of the cartilage and associated periosteum varied with the samples. Most samples exhibited consistent receipt of staining medium within the cartilage covering of the condylar head; however there was inconsistent staining of the periosteum inferior to the condylar head covering the ascending ramus. This was reflected in variations in the segmentation of the cartilage and periosteum. Consequently this may influence the volumetric measurements of cartilage/periosteum values.

11.4.3 Segmentation methodology

To reduce the inconsistency in selecting cartilage or bone gray scale values, it was decide that all samples be analysed with a standardised protocol of expanding the selection area by the same amount in each sample. Expansion of a selected area would result in the selected area increasing in volume one pixel in each of the three dimensions with each expansion step.

Expanding the selection four times allows favourable selection of the total bone/cartilage/periosteum volume. This would result in consolidation of separate areas selected by the segmentation tool after the selection of an appropriate grayscale value within the trabeculae of the bone. It would also reduce or obliterate the large voids present in the marrow of the condylar head, allowing the selection of fine trabeculae present in these areas. Subsequently, a level of noise (i.e. unrelated data) was included in all measurements, as the expanded selection now increased in volume peripherally beyond the cartilage/bone/periosteum surface. But this was consistent throughout all samples, allowing direct comparisons between samples. This method was used for all total volume measurements (Appendix 17.4).

A similar rationale was used in the selection of cartilage/periosteum from the total volume selected previously. Expanding the selection by three times allowed for a similar consolidation of areas of selected by the segmentation tool. Notably, this study did not look at taking the absolute value of any volume. Absolute cartilage volumes were not possible due to the non-specific nature of the staining medium in regards to cartilage and periosteum. Instead, it was the intention of this study to examine the relationship that occurred between groups and between different days of experimentation with a constant methodology error in place with all measurements. This would allow direct comparisons to each sample. The method error analysis further validates the high reliability of measurements.

11.5 Future application of this methodology

Future investigations of gadolinium based imaging in MicroCT should consider the disarticulation of the mandible from the rest of the rat head. Disarticulating the condylar head

may reduce the immersion time sufficiently and possibly closer to that achieved by Cockman et al [100]. It would also allow reliable segmentation of the condylar cartilage, by reducing the noise seen in this investigation. Images would then directly depict the condylar cartilage and its adjacent subchondral bone. Additionally, direct visual comparison of the condylar head to 3D reconstruction would be possible to check the consistency of the condylar cartilage.

11.5.1 Effects on Staining

This would allow direct immersion of the condylar cartilage in the gadolinium staining medium. A disadvantage of this current protocol is that images produced demonstrated patchy areas of pickup in certain surfaces, predominantly on the medial surface. This may have been the result of limited diffusion into this area by the staining medium. Direct immersion of condylar cartilage would complete a number of prerequisites simultaneously. Firstly, the inconsistent selection of cartilage/periosteum and bone/cartilage/periosteum would be improved. This would improve receptiveness to the staining medium, improving the image consistency. Secondly, this would eliminate the variations in imaging contrast between samples as a result of scanning, reconstruction or reorientation processes. Thirdly, address the inability to select a constant grayscale threshold during the segmentation process as no single anatomical structure has a defined grayscale value. And finally allow consistent and replicable measurements to be made and allow comparisons to be made.

11.5.2 Effects on segmentation

It was an initial aim of this investigation to examine the condylar head of the mandible in relationship with the associated anatomical structures. Efforts were made to ensure that the

tempormandibular joint was undisturbed during specimen preparation. However, after scanning and 3D reconstructing samples, it was clear that some samples exhibited altered condylar head positions in relationship to the articular fossa. Consequently, segmentation of the condylar head away from the overlying zygomatic arch proved difficult. These samples demonstrated a direct contact with the overlying zygomatic arch or extremely close proximity. This made separation of the two structures were very difficult and impossible in some cases.

11.5.3 Effects of image reorientation

Another issue in this investigation was software reorientation. This has the potential to "contrast crush" the image. "Contrast Crush" occurs as a result of the reorientation of the slices. A side effect of rotation of slices maybe the condensation of grayscale values, resulting in the loss of contrast between high contrast and low contrast structures. Examination of the grayscale values before and after rotation noted no differences.

11.5.4 Future applications

Traditional 2D studies of coronal and sagittal slices as seen in histological slices, may be assessed morphologically via this non-destructive contrast media enhanced MicroCT method. Though it may not give the same information as immunohistochemistry or histological studies, gross morphological changes can be visualised two dimensionally and compared to changes three dimensionally within the sample. Future investigations may examine the changes within the condylar head of the same sample, view macroscopically via 2D and 3D

non-destructive methods. The same sample may then be examined by histological or immunohistochemistry means more thoroughly via more destructive histological sectioning.

11.5.5 Summary of Experimental Methodology

Many of the issues related to imaging and analysis of the condylar cartilage and associated underlying bone structure could be resolved at the specimen preparation stage. All previous studies examining the use gadolinium based staining mediums. This allowed for diffusion of the media into the cartilage *in vitro* [100] and with the presence of a living body system, rapid diffusion of the media in *in vivo* studies. [14] Imaging of the condyles would been more reliable if the condylar head was disarticulated from the fossa. This would allow direct immersion of the condylar cartilage and periosteum with the gadolinium chloride. Reduced immersion time for acceptance of the media on all surfaces would result in improved staining media pickup and visualisation. Other advantages of condyle disarticulation include:

(1) Allow easier positioning of the sample within the Micro-CT machine.

(2) Allow a reproducible position of the condylar head, reducing the need for software manipulation to reorientate the samples. Construction of a sturdy, specially designed jig is required to assist in reproducing the ideal orientation in all samples and allow for rigid fixation of the specimen. This would minimise the amount of movement of the sample as it rotated within the MicroCT machine, hence minimising the need to compensate for movement as well as issues with alignment during orientation.

(3) Allow for improved MicroCT imaging parameters as the associated skeletal, muscular and ligamentous structures would no longer influence the x-ray beam penetration.

(4) Reduce the need to alter the selection area via expansion, possibly allowing true volumetric measurements to be carried out of the total volume of the sample and the cartilage/periosteum.

(5) Ensure reproducibility of the 3D reconstruction and segmentation as no noise will be present peripheral to the condylar head.

(6) Allow direct macroscopic examination of the specimens to validate the 3D reconstructions.

Nevertheless, this investigation highlighted a new direction for orthodontic/orthopaedic research in the examination of changes in the condylar cartilage of Sprague Dawley rats. It emphasises the need to assess changes in a 3D sense rather than via individually selected slices. Though, this method requires further refinement, it does provide the foundations for a robust and reproducible method of analysis.

11.6 Future directions

11.6.1 The relationship between Gadolinium and proteoglycan concentrations

Kallioniemi et al suggested that in intact articular cartilage, proteoglycan concentration typically shows uneven distribution demonstrating lower concentrations at superficial cartilage compared with the deeper tissue layers. [14] It was also demonstrated the use of a gadolinium based contrast agent (gadopentetate) within articular cartilage, after complete

diffusion. Cockman et al [100] also suggested that the correlation between MicroCT intensity and proteoglycan content was probably a function of the concentration of the gadolinium solution. It has been speculated that greater concentration of gadolinium could increase the ability to distinguish between more subtle variations in proteoglycan content with MicroCT. However, the practicality of this would be limited by the solubility of the gadolinium chloride solution. [100] The concentration of gadolinium needed for MicroCT studies of cartilage is open for debate and is governed by whether it is used *in vitro* or *in vivo*. As the concentration of gadolinium increases, the X-ray attenuation increases. Accordingly, this would enable better visualization of cartilage and may improve the sensitivity to variations in proteoglycan content. Delivering a high concentration of gadolinium to cartilage *in vivo* could be achieved by intra-articular or intravenous injection. Currently, 200mM gadolinium chloride solution is limited to *in vitro* examination of rodents as this concentration would be lethal to mice and rats. [129,130] Therefore MicroCT, using a high concentration gadolinium probe, will have its greatest applicability for terminal studies. The relationship between gadolinium and proteoglycan concentrations is applicable in the diagnosis of proteoglycan-related cartilage degradation. Degradation of cartilage allows for significantly increased penetration of the contrast agents possible, however information regarding the pharmcodynamics of differing staining media concentration into cartilage is future investigation. [14]

Advances in contrast agents have entered the realm of molecular imaging. Gadolinium based nanostructures have been developed that have unique properties on a nanomolecular level and have superior performance to traditional MRI contrast agents. These show promise for molecular imaging and other advanced applications. [91] The use of nanotechnology allows the production of molecular agents that are best suited for cellular uptake, biocompatibility and eventual elimination from the body. [91] They also hold promise in intracellular imaging as

these agents have been shown to translocate into the interior of cells with minimal cytotoxicity. [91] It has been suggested that molecular imaging is a new frontier for diagnostic medicine. It will strengthen diagnostic accuracy of existing image modalities and their interpretation by probing unique biological signatures or subcellular processes that are at the cause of disease. [91]

Hence, further investigations on the concentration of gadolinium based staining media; its pharmacology and the application of molecular imaging may hopefully result in the clinical applications of these staining media in the diagnosis of cartilaginous pathology associated with the temporomandibular joint.

11.6.2 Future investigations into the process of chondrogenesis

Intense studies have been carried out in the medical orthopaedic literature in the understanding of the contribution of articular cartilage and osteoarthritis.[20,29,101,103] However, the process of chondrogenesis in both articular cartilage and condylar cartilage, is not well understood. Further understanding in the process of chondrogenesis is not only relevant for degenerative diseases that affect both articular and condylar cartilage, such as osteoarthritis but also relevant for normal growth and development. Evidence is beginning to emerge that osteoarthitic chondrocytes are quite metabolically active and reinitiate the synthesis of some proteins that are characteristics of early developmental stages. [29] Furthermore, articular cartilage has a unique ability to physiologically adapt to its environment, by altering its structure and composition to meet the physical demands of the body. [21]

An understanding of cartilage differentiation and development may provide guiding principles for tissue engineering of cartilage, and may, therefore, play a part in new therapies for degenerative cartilage diseases. [29] This has implications in orthodontics, particularly those suffering from degenerative juvenile or rheumatoid arthritis of the TMJ, as well as other achondroplastic disorders, not only affecting the condylar cartilage but other cartilages of the craniofacial system.

11.6.3 Future studies with MicroCT and condylar head

The development of a MicroCT, capable of 3D reconstruction at a resolution of 5-50μm, has allowed evaluation of changes in trabecular subchondral bone. [101] It has been postulated that the structural properties of subchondral bone may play a role in the degeneration of articular cartilage, and bony changes may even precede cartilage degeneration. [103] Dedrick et al [101] using MicroCT in assessing early bone changes, as depicted by alterations in trabecular subchondral bone in an experimental osteoarthritis model of a guinea pig hind limb, found that these bony changes can be found as early as the histological changes in cartilage. They found that cartilage matrix alterations can be detected by 16 weeks using metachromatic stains of the cartilage and surface alterations seen by 32 weeks. However, at 16 weeks, subchondral bone demonstrated greater trabecular separation and smaller individual trabeculae compared to controls. They concluded that as early as 8 weeks, differences in the trabecular bone between experimental and controls can be detected, which changes through time. They also noted that these bony alterations can be detected as early as changes in the cartilage. In another MicroCT evaluation of normal and osteoarthritic bone structure in

human (cadaver) knee specimens, Patel et al [103] commented that it is currently unknown if the trabecular changes of the subchondral bone precede or follow changes in the cartilage. [103]

This study demonstrated that there was not an increase in the volume of cartilage as aniticipated with peak growth and appliance placement. Degeneration was also evident in this study. Future investigations could examine changes in the bony trabecular of the underlying subchondral bone over the time period of appliance placement. Further investigations are required into the interrelationship between changes in condylar cartilages and the underlying subchondral bone due to orthopaedic intervention.

11.7 Conclusions

This study demonstrated that the condylar cartilage of Sprague Dawley rats can be successfully visualised and quantitatively measured via enhanced MicroCT. The following conclusions can be made:

(1) Alterations in loads applied to the condylar head resulted in functional adaptations within the condylar head. This was represented as conformational changes in the shape of the condyle.

(2) Anterior displacement of the condylar head resulted in significant remodelling changes in contrast to normal and posterior displacement of the condyle.

(3) Alterations in joint function results in cartilaginous changes different than expected of normal condylar growth.

(4) Differences between anterior and posterior displacement groups were not significant however obvious trends were present and require further investigation.

(5) Though further refinements are required to improve this technique, this study has shown a new viable alternative to measuring condylar cartilage change.

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13 Figures

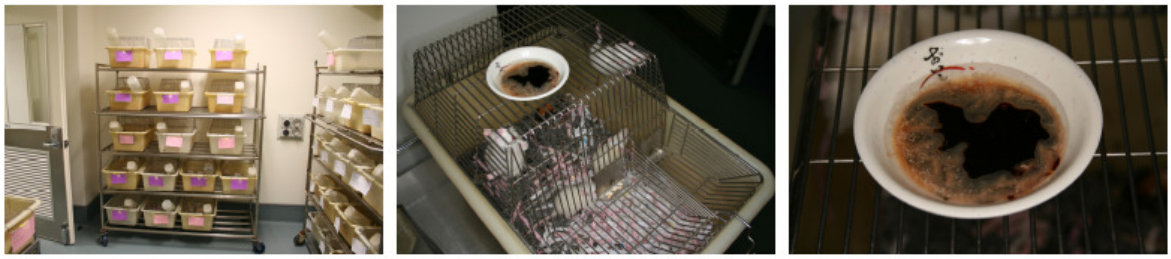


Figure 1 - Animals were housed in a controlled room (left). Cages were maintained with paper based bedding (middle). Food preparation occurred daily with Nutrigel placed in a dish. Water was added to aid consistency (right)

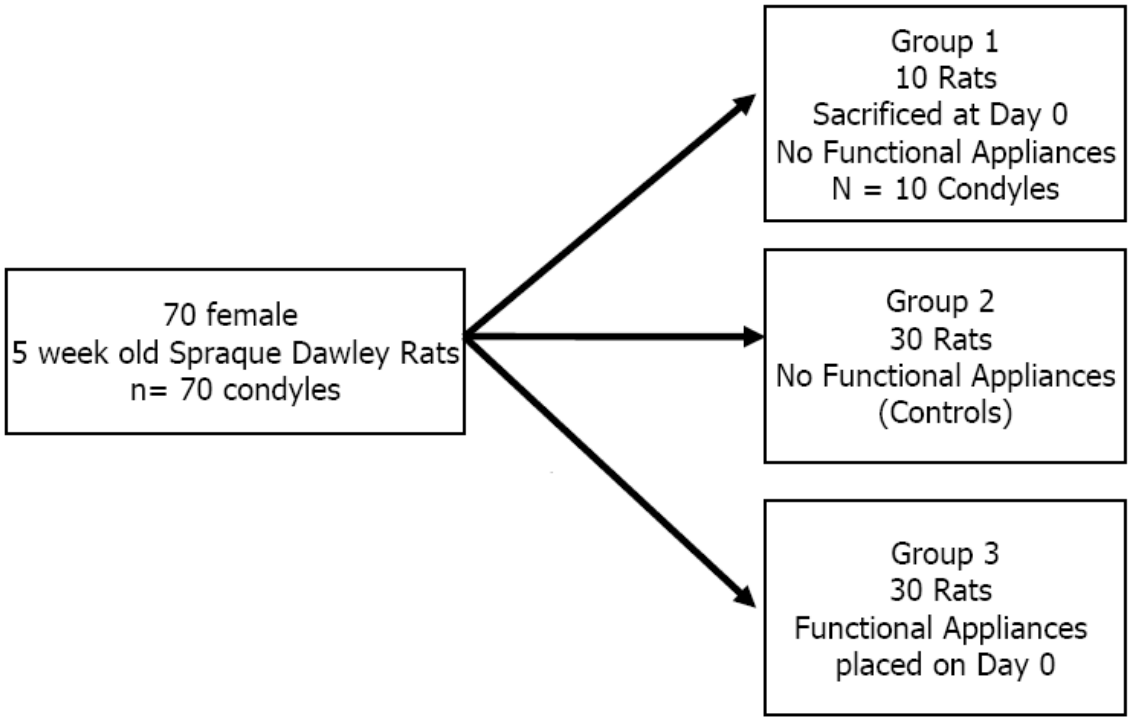


Figure 2 – Sample Distribution on Day 0

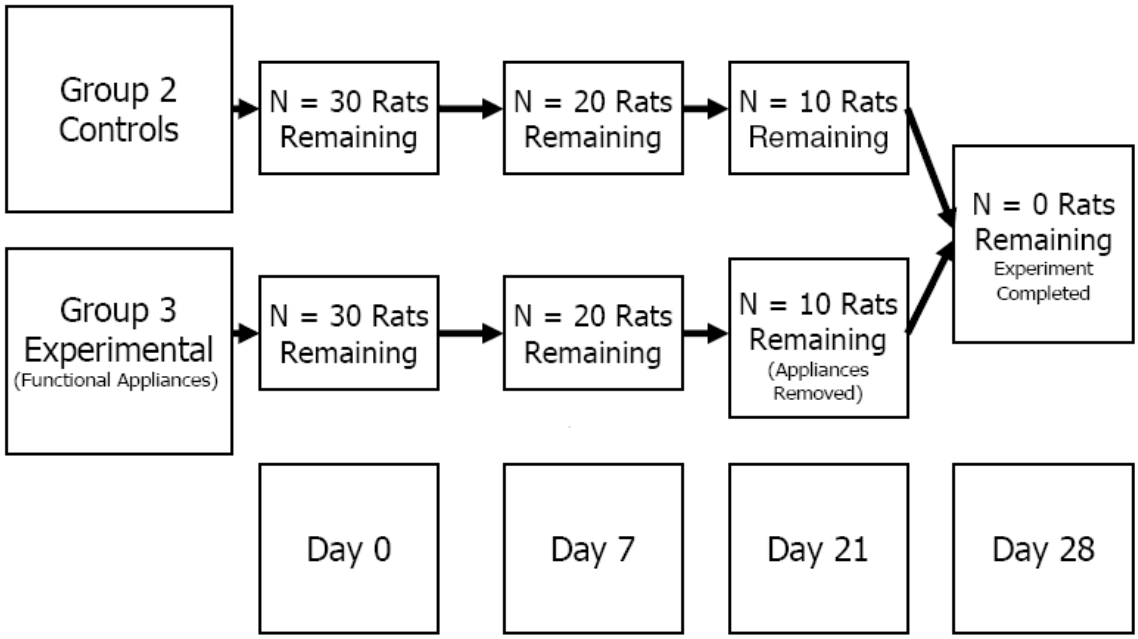


Figure 3 - Study Design between Days 0-28

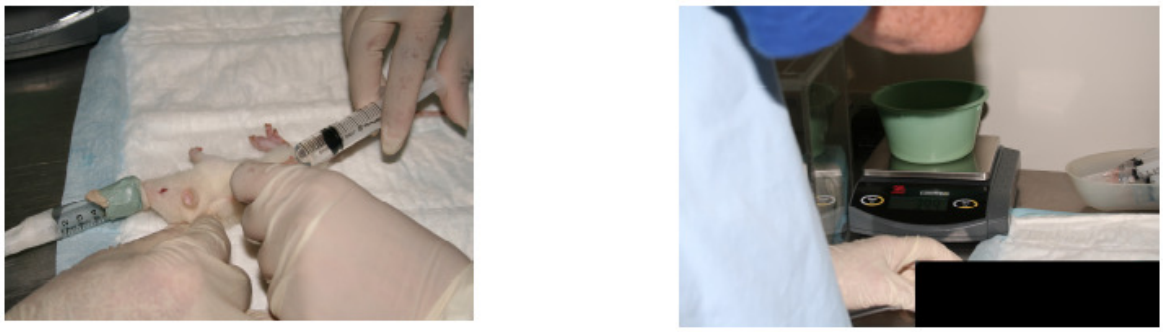


Figure 4 - Experimental animals receiving inhalational and general anaesthesia (left) and being weighed (right) prior to appliance placement

Figure 5 - – Lower incisors (left) of experimental animals being primed with SEP (3M ESPE) and Paediatric Crown formers filled with Dental composite, placed over the lower incisors (right)

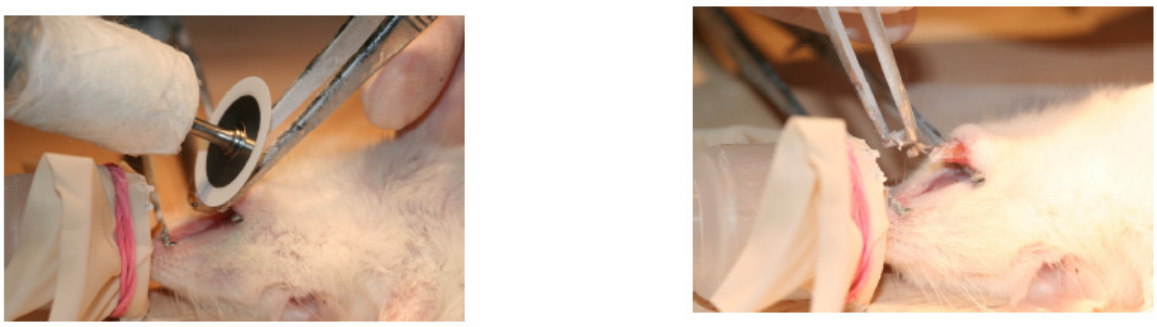


Figure 6 - Day 28 experimentals had appliances removed at Day 21. Oscillating disk was used to carefully remove the crown former (left), before the remaining composite was easily dislodged (right)

Figure 7 - Anterior Jaw position with appliances in place (left) and after removal of appliances (right)

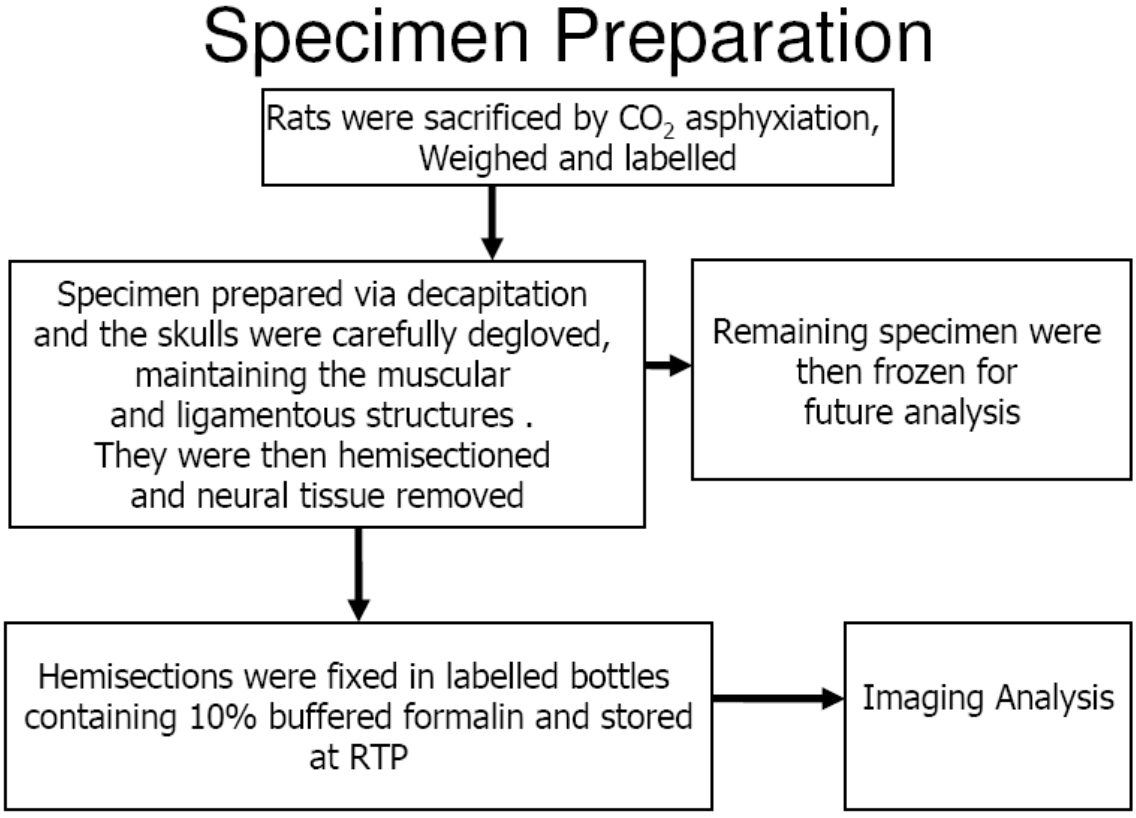


Figure 8 - Specimen preparation

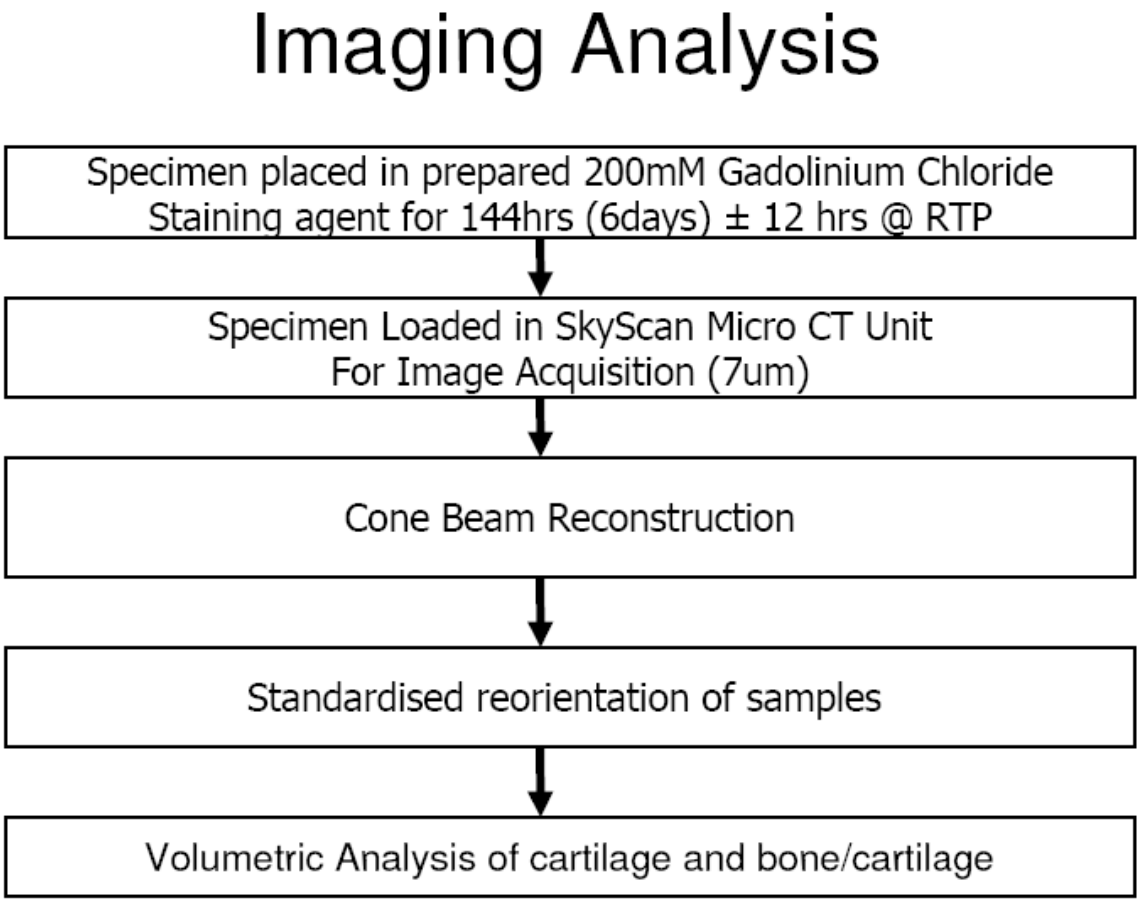


Figure 9 – Pre-Image analysis

Figure 10 - Right hemisection removed from 10% buffered formalin (left) and sample dried prior to scanning (right)

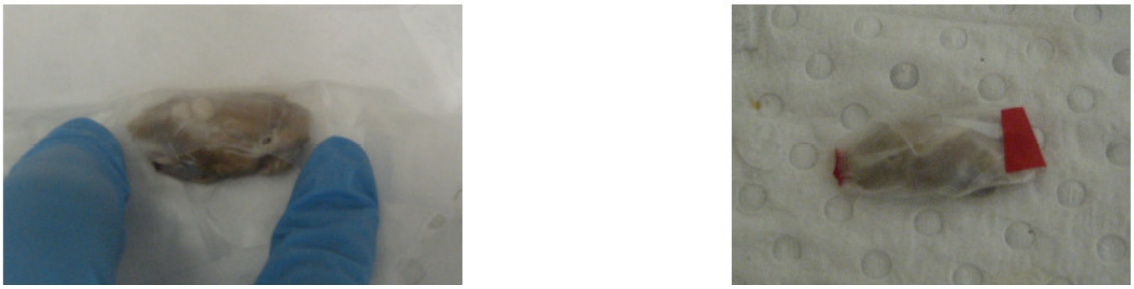


Figure 11 - sample was then sealed in paraffin film (left) and secured fastened with electrical tape (right)

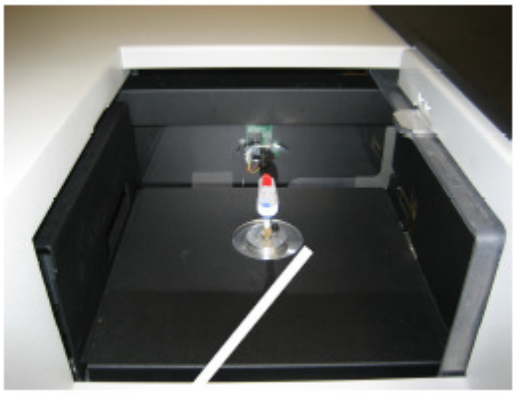


Figure 12 - Specimen was securely fastened in a styrofoam jig and placed within the SkyScan MicroCT scanner

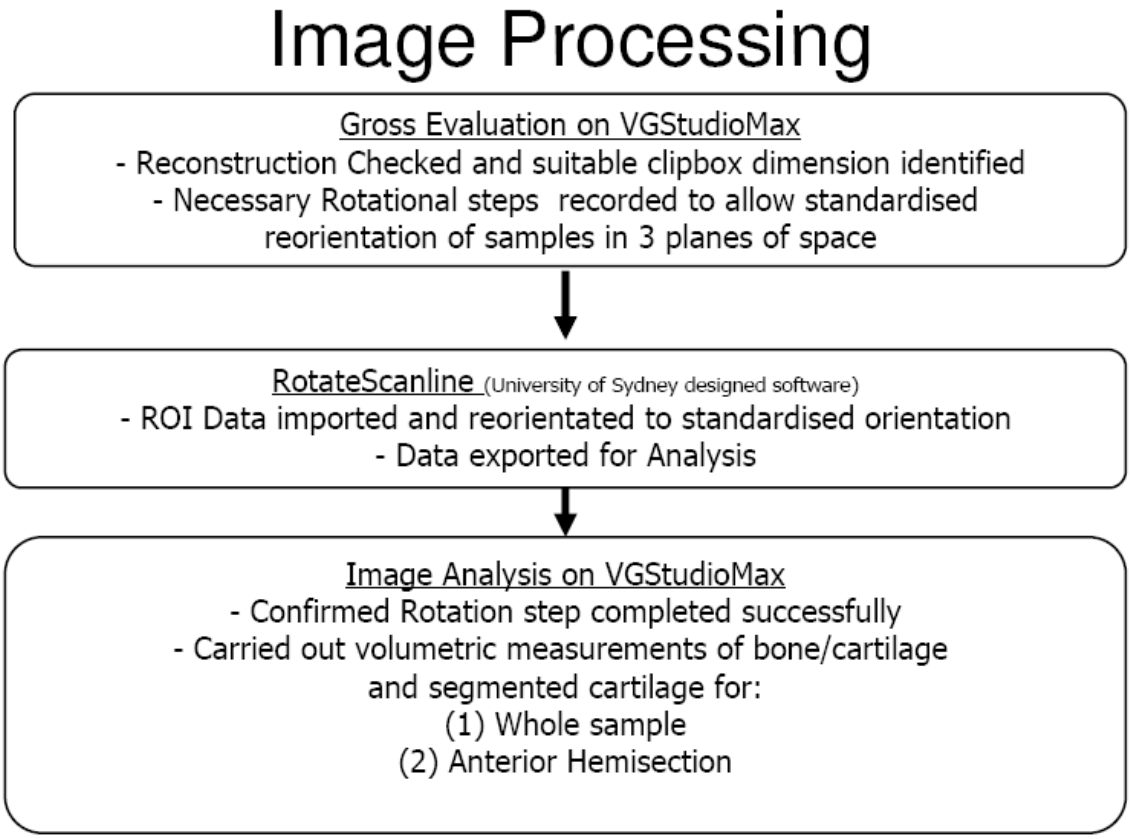


Figure 13 - Image Processing pathway

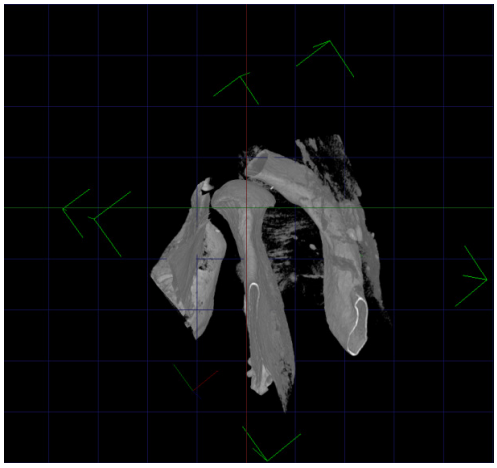


Figure 14 - Alignment of coronoid notch with the vertical (red) axis – Viewed anteriorly

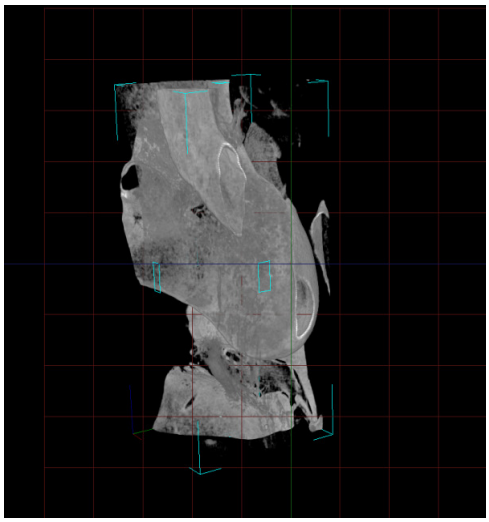


Figure 15 - Alignment of lateral pole of condylar head to another axis (green) axis - viewed laterally

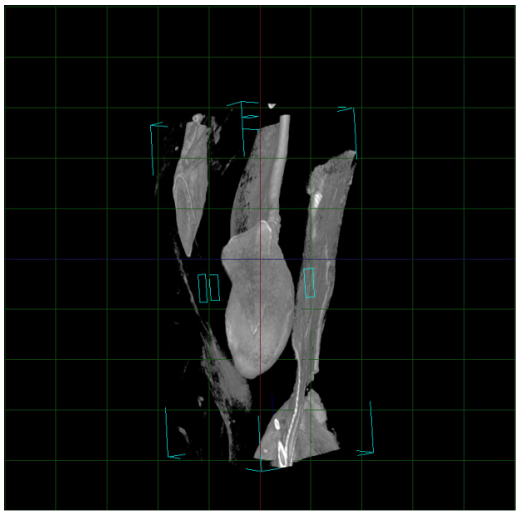


Figure 16 - Alignment of condylar head and coronoid notch parallel to an axis (red) - geometric centring of the condyle before standardised angulation to MSP

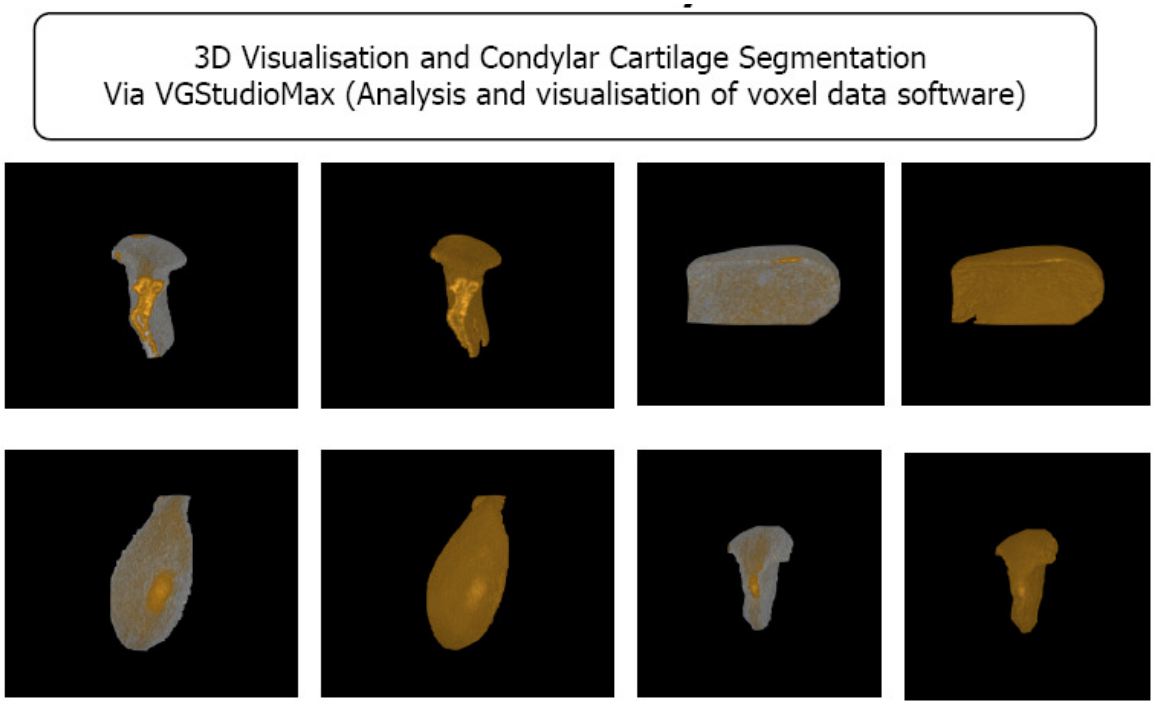


Figure 17 - Example of the segmentation of Cartilage volume (Orange) from Total Volume (Orange/Grey) - viewed from multiple angles

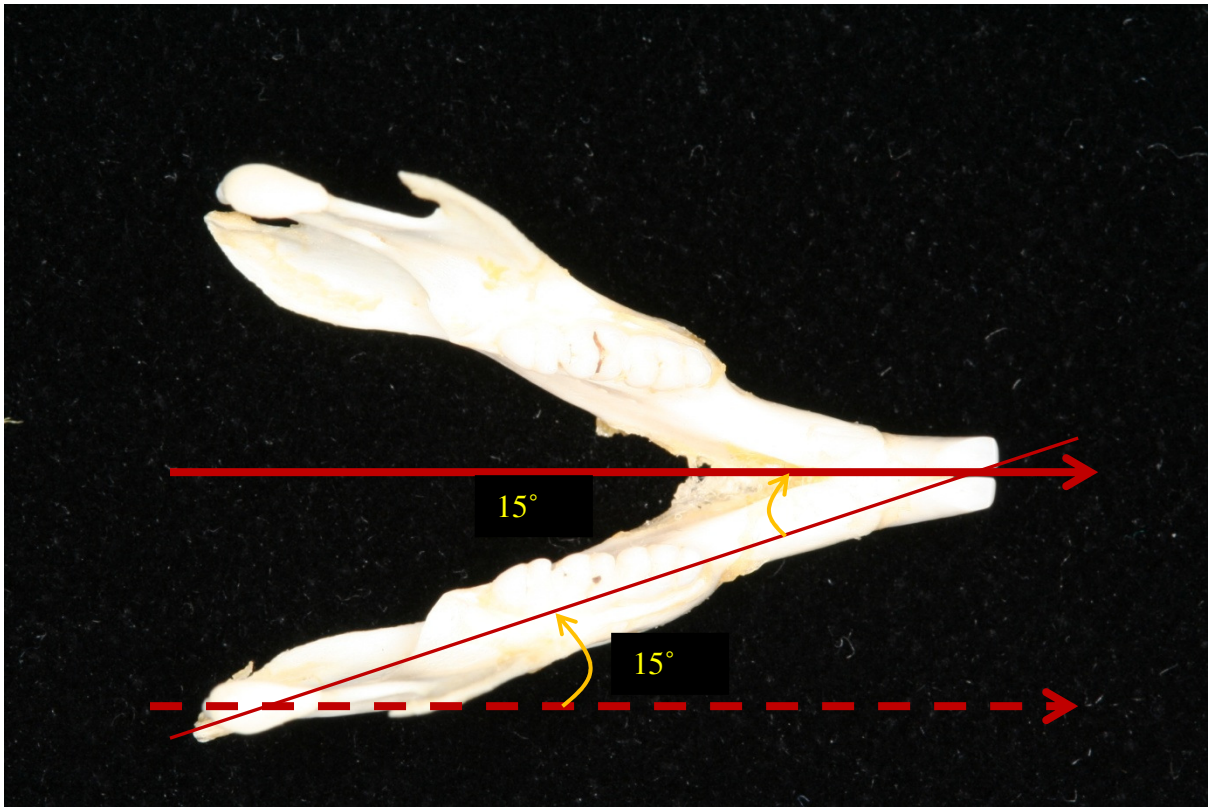


Figure 18 – Arbitary condylar axis angle (15˚) taken from a Dry 5 week old Sprague Dawley Rat Skull

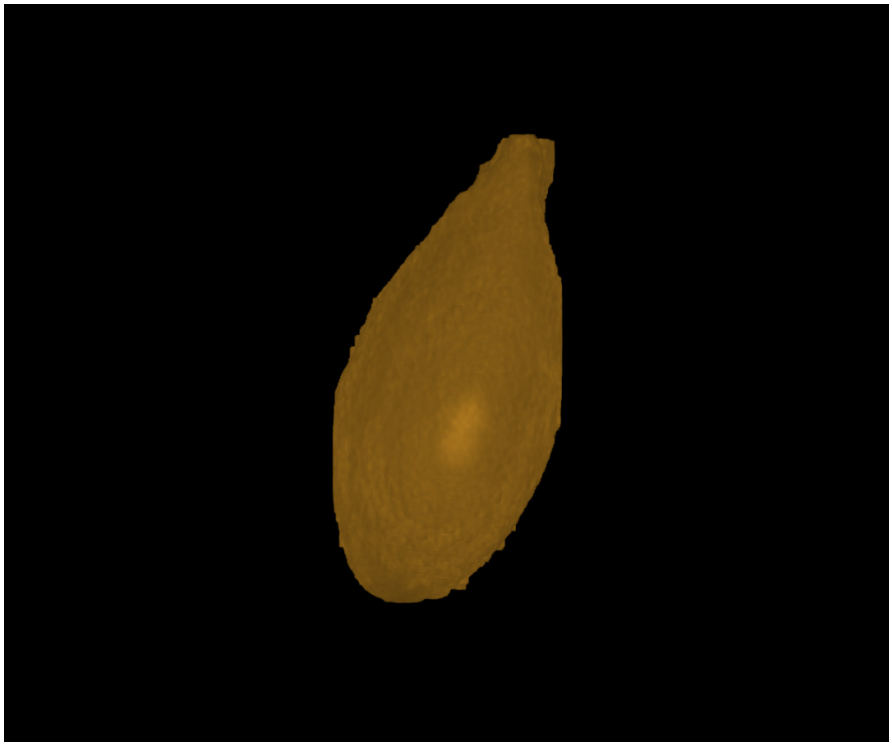


Figure 19 - (above) - Control (Day 0 - GLD005) - Superior view demonstrating a symmetrical ovoid shape

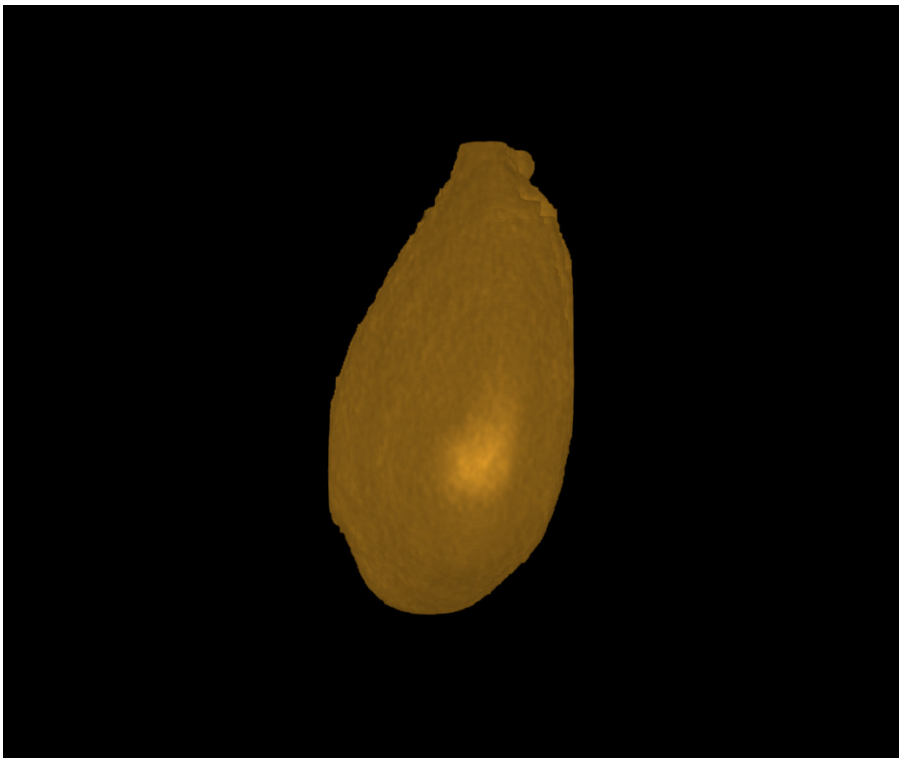


Figure 20- (above) - Control (Day 0 - GLD073) - Superior view demonstrating a tear drop (posteriorly weighted) shape

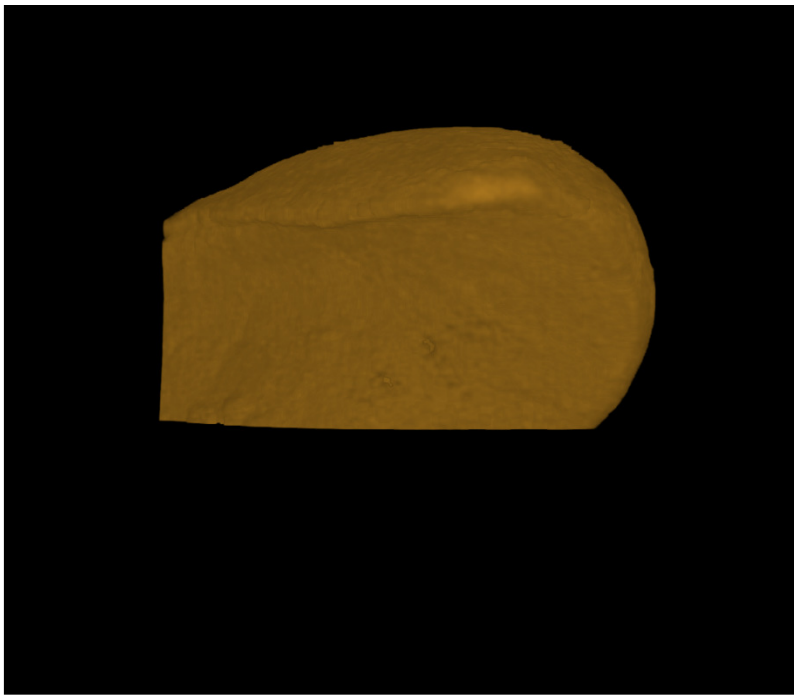


Figure 21- (above) - Control (Day 0 - GLD005) - Lateral view demonstrating the posterior boundary of the condylar head and its convexity. The lateral pole demarcation is also evident

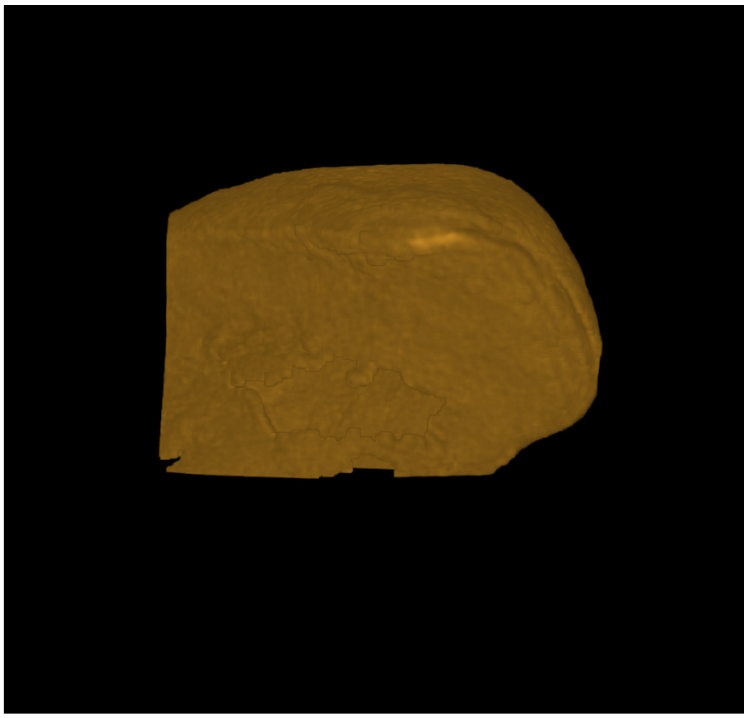


Figure 22- (above) - Control (Day 0 - GLD075) - lateral view demonstrating the posterior boundary and its pointed shape

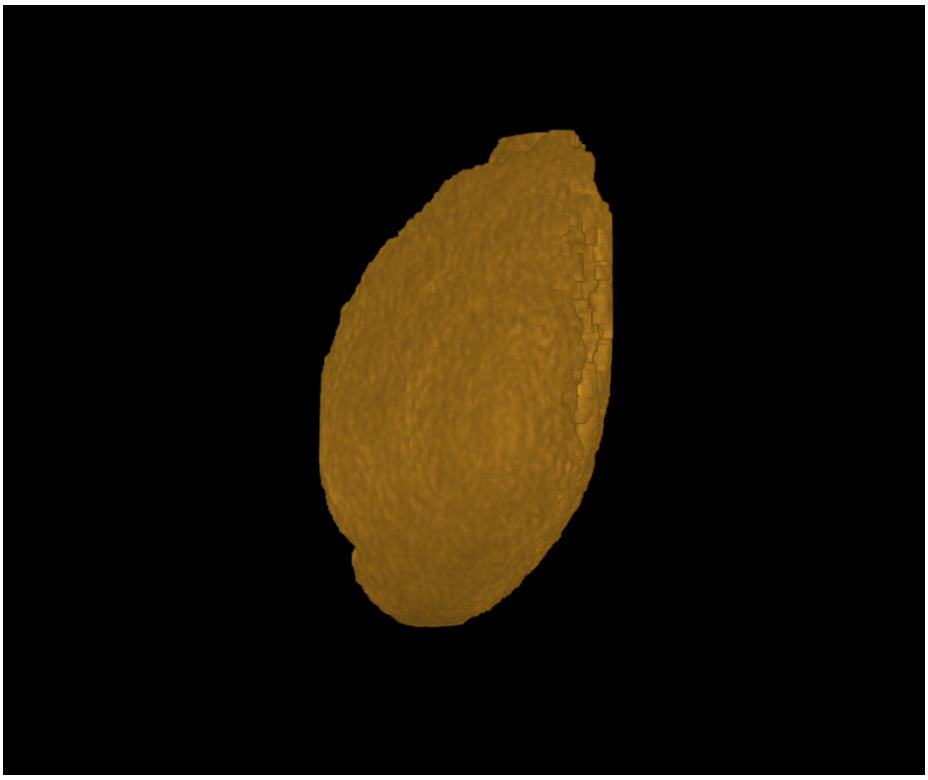


Figure 23 - (above) - Control (Day 7 - GLD007) - Superior view demonstrating a more centrally weighted (square/rectangular) shape

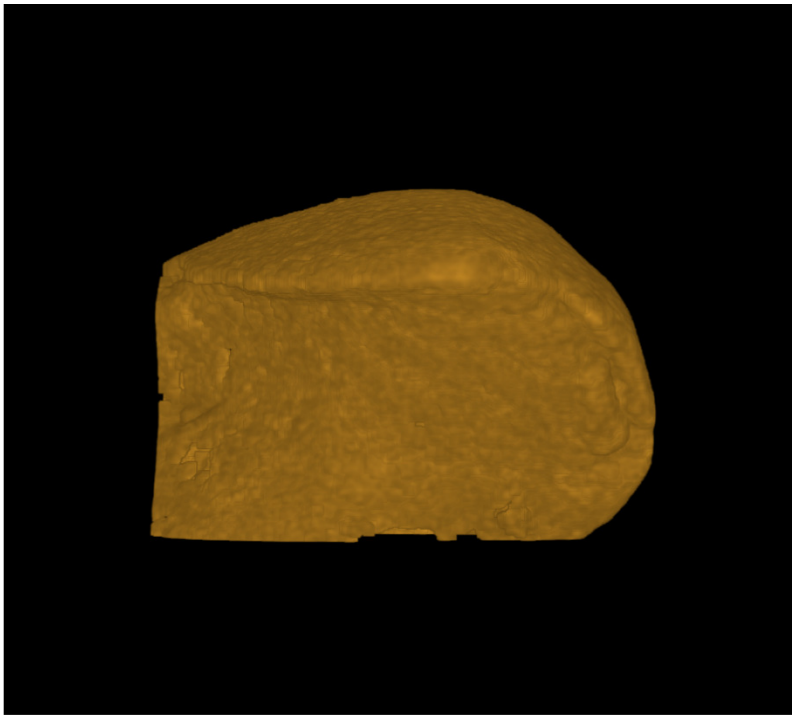


Figure 24 - Control (Day 7 - GLD007) - Lateral view

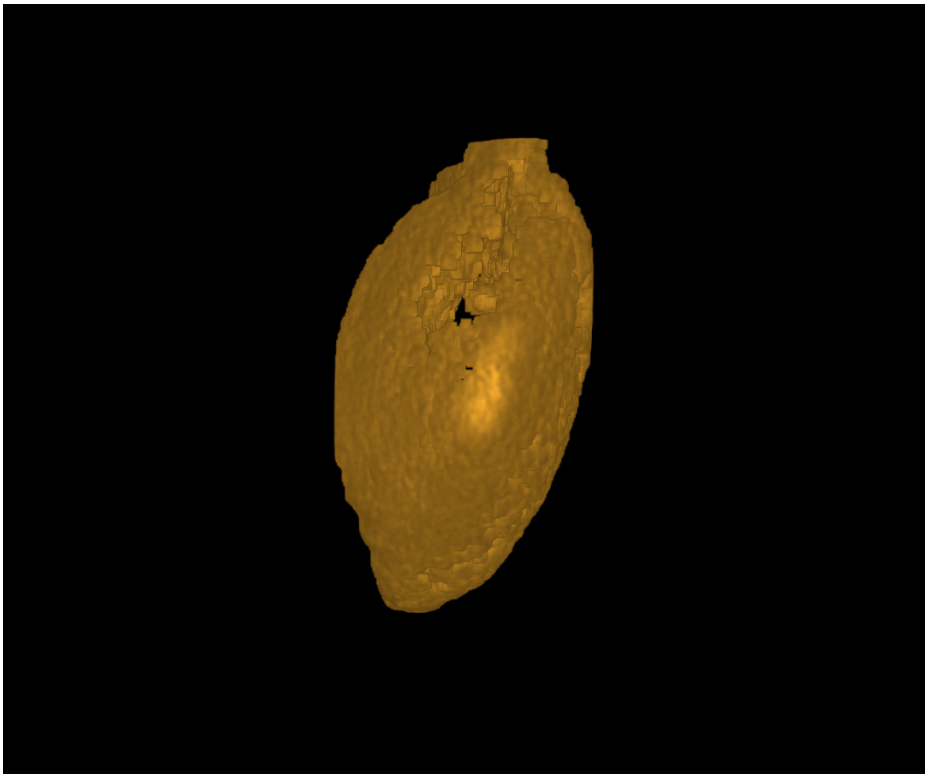


Figure 25 - (above) - Control (Day 21 - GLD013) - Superior view demonstrating a more centrally weighted (square/rectangular) shape

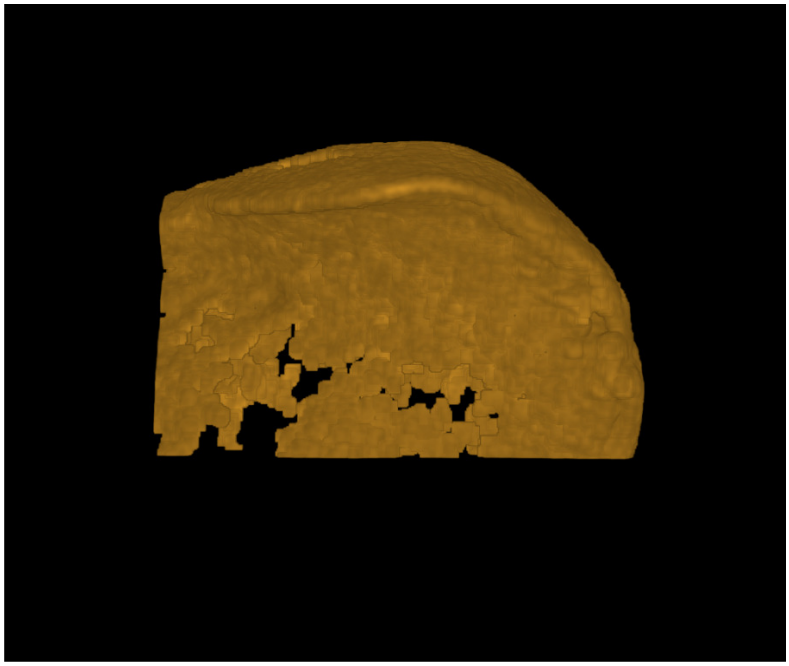


Figure 26 - (above) - Control (Day 21 - GLD013) - Lateral view

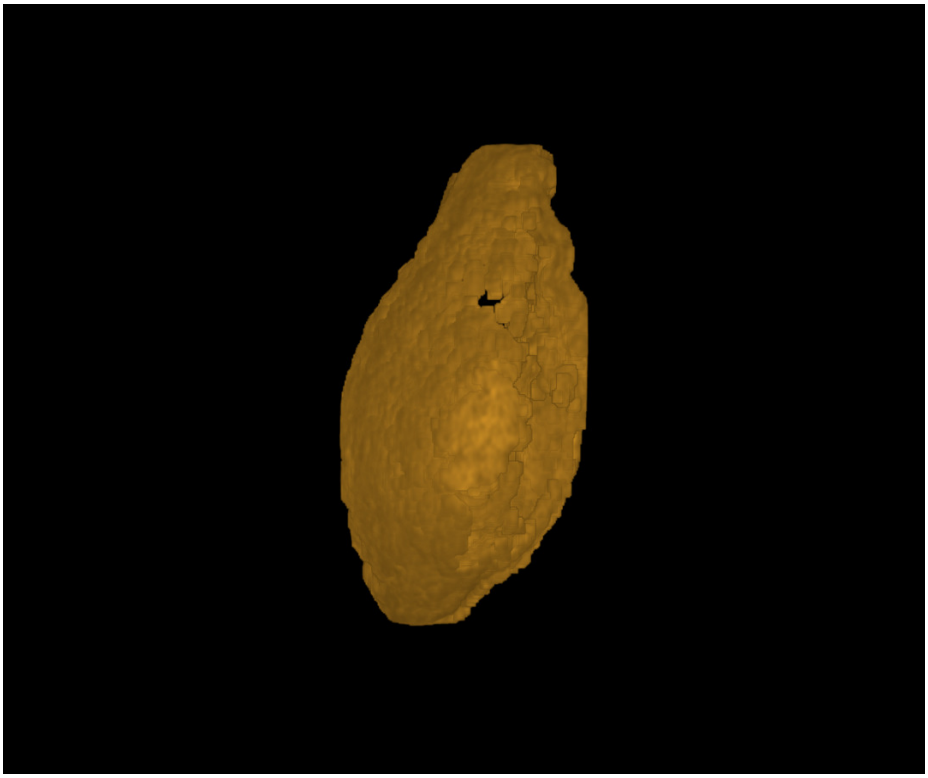


Figure 27- (above) - Control (Day 28 - GLD089) - Superior view demonstrating a more centrally weighted (square/rectangular) shape

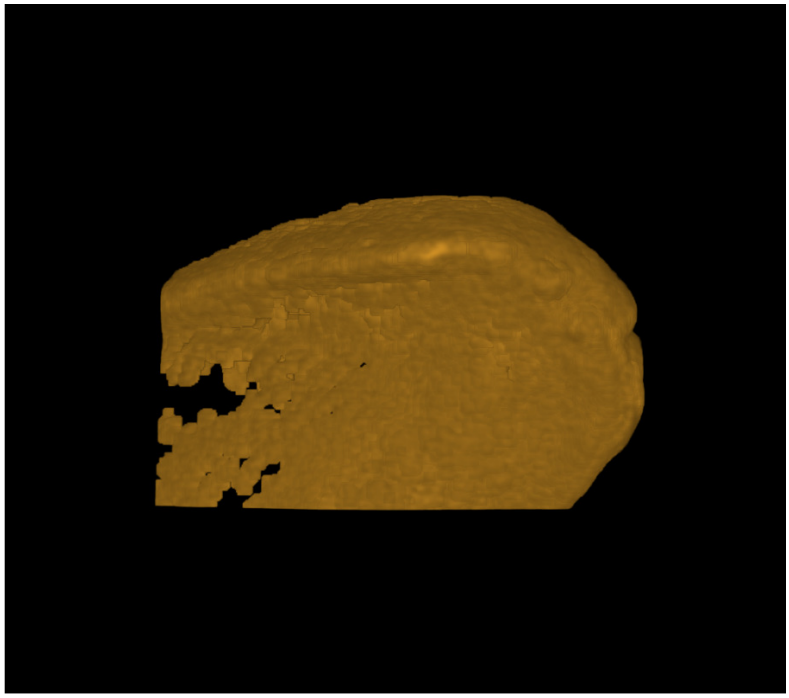


Figure 28 - (above) - Control (Day 28 - GLD089) - Lateral View

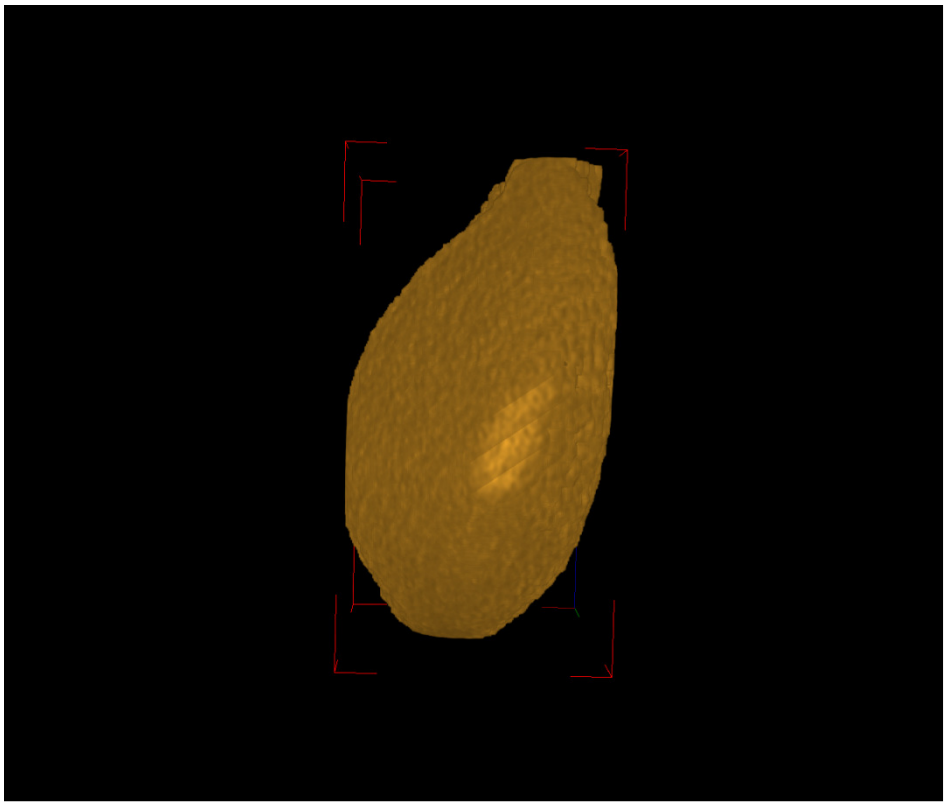


Figure 29 - (above) - BWD (Class II Day 7 - GLD023) - Superior view

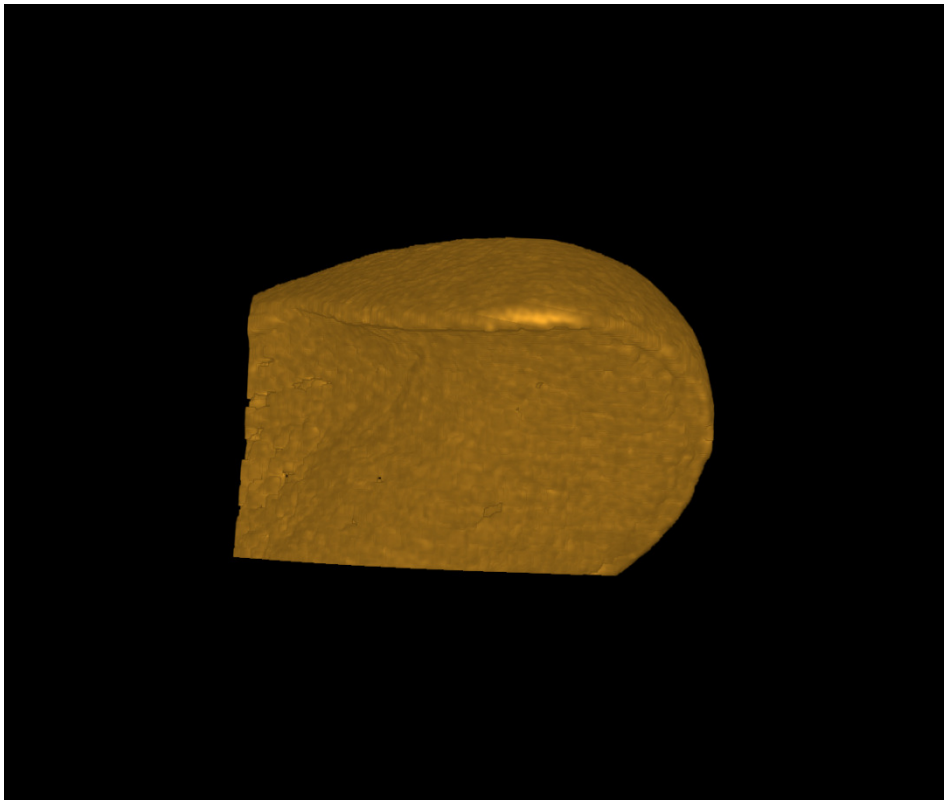


Figure 30 - (above) - BWD (Class II Day 7 - GLD023) - Lateral view

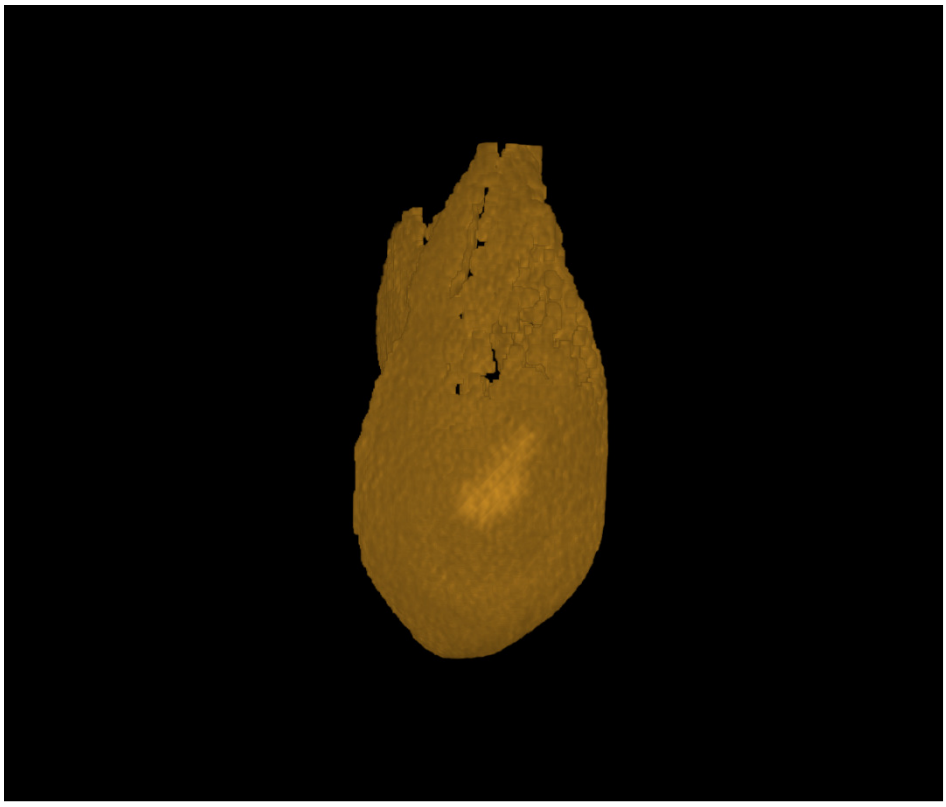


Figure 31 - (above) – BWD (Class II Day 21 - GLD035) - Superior view

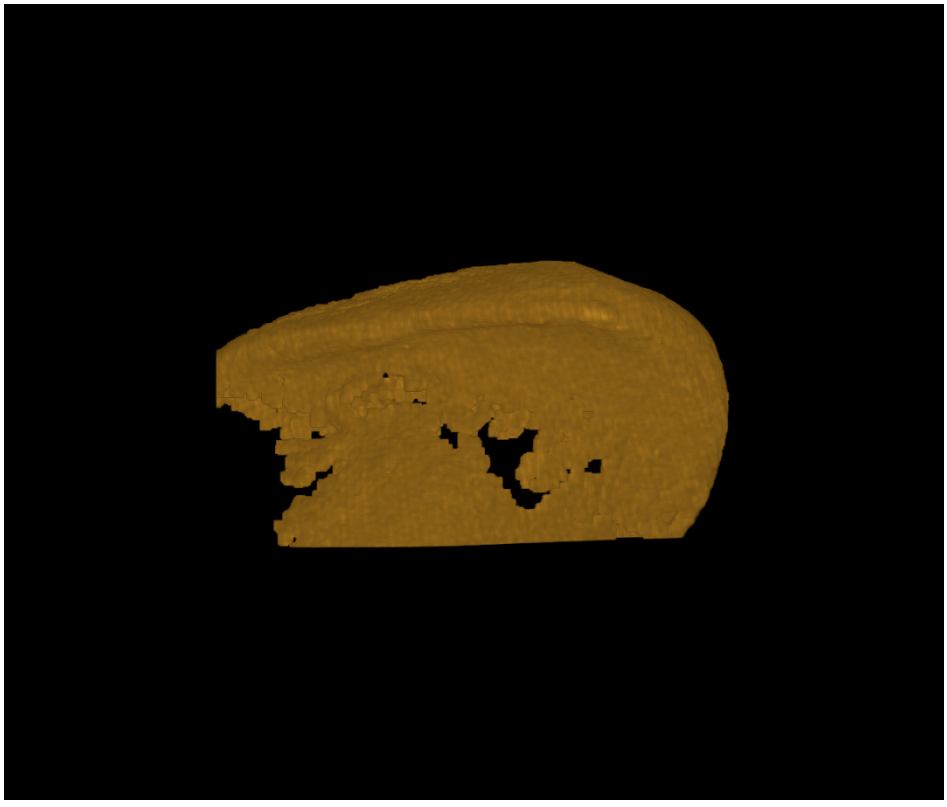


Figure 32 - (above) – BWD (Class II Day 21 - GLD035) - Lateral view

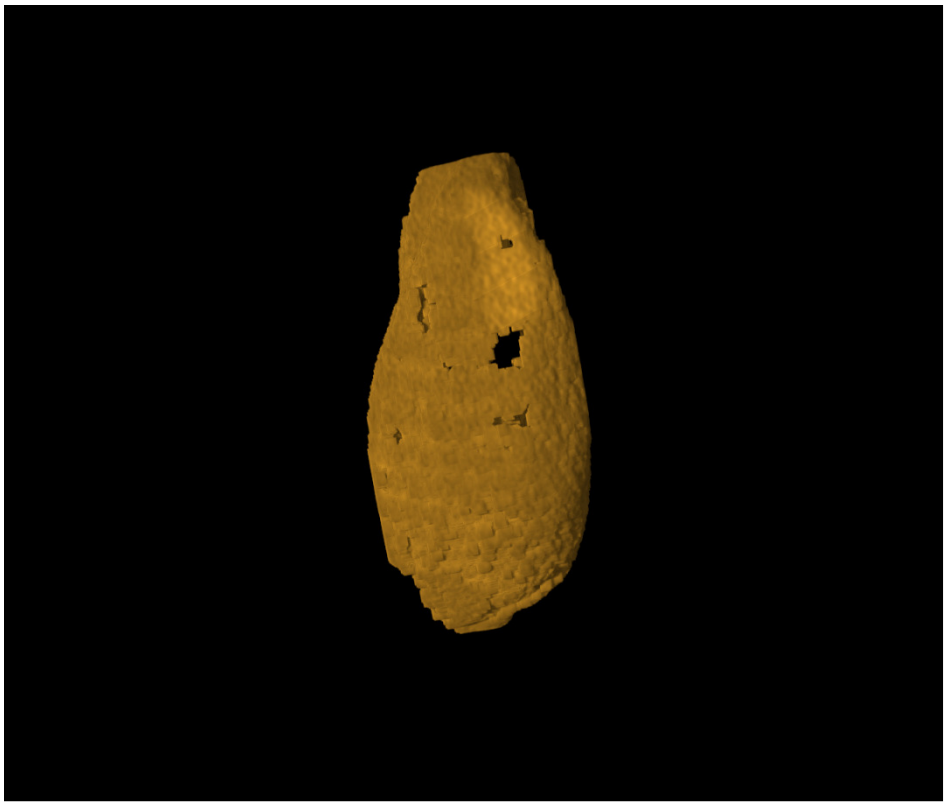


Figure 33 - (above) – BWD (Class II Day 21 - GLD103) - Superior view

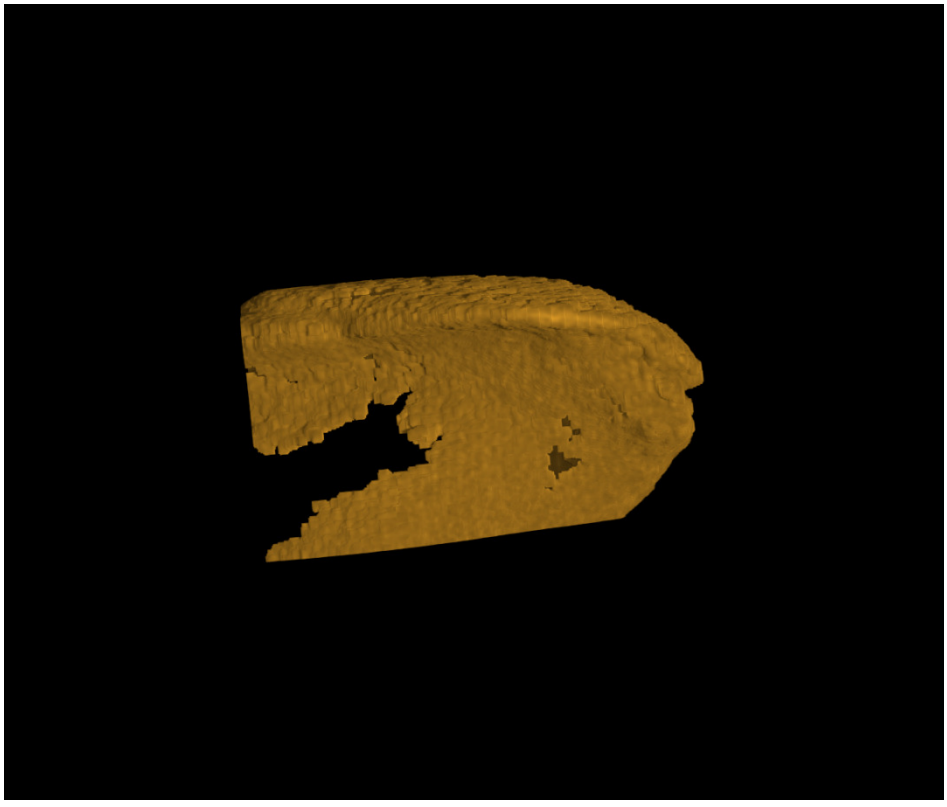


Figure 34 - (above) – BWD (Class II Day 21 - GLD103) - Lateral view

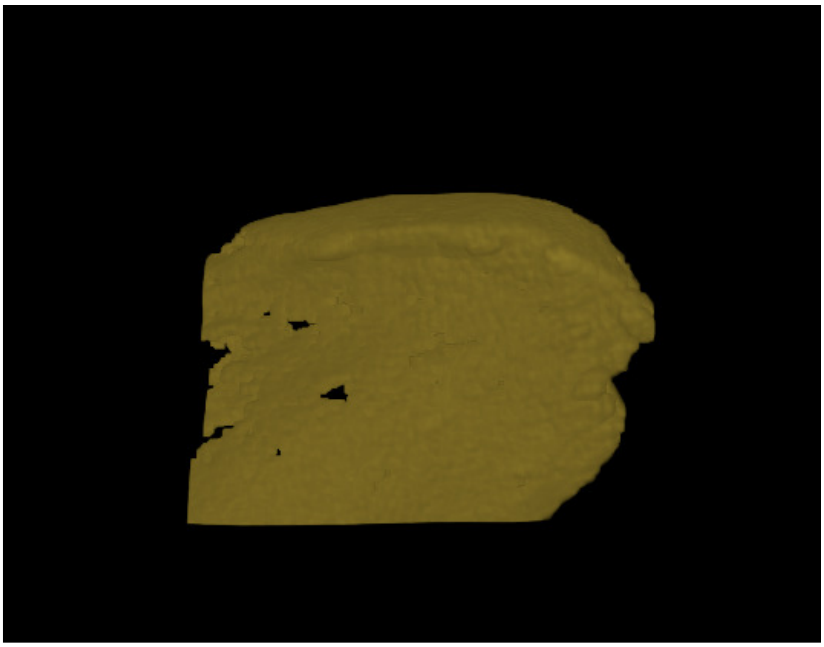


Figure 35- (above) – FWD (Class III Day 21 - GLD101) - Lateral view

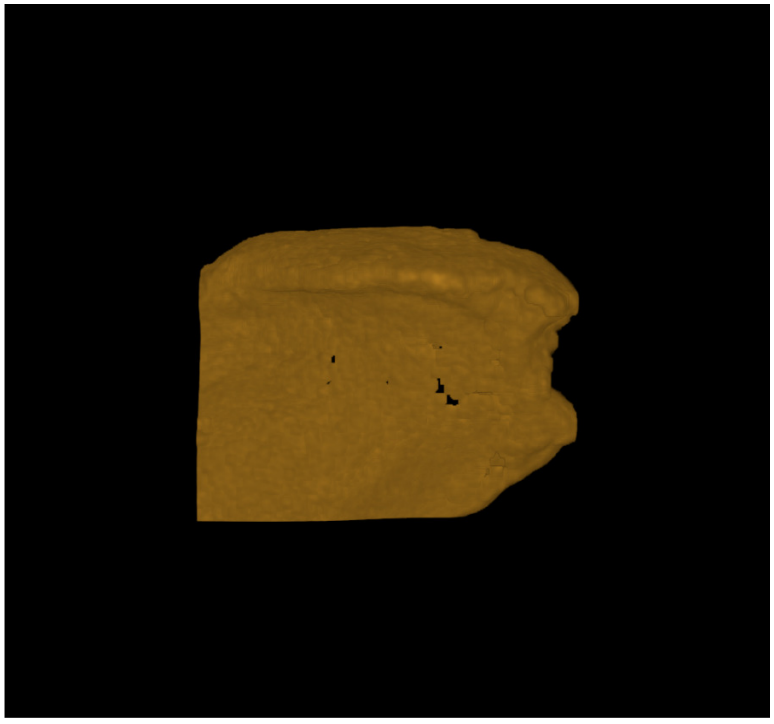


Figure 36 - (above) – FWD (Class III Day 21 - GLD032) - Lateral view

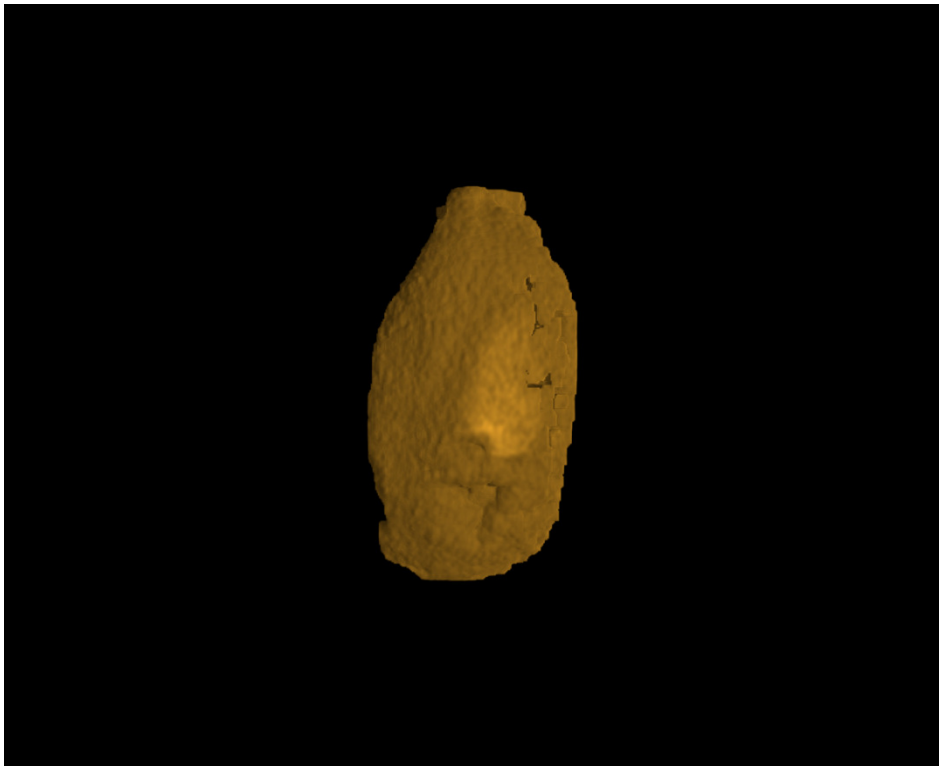


Figure 37 - (above) – FWD (Class III Day 21 - GLD032) - Superior view

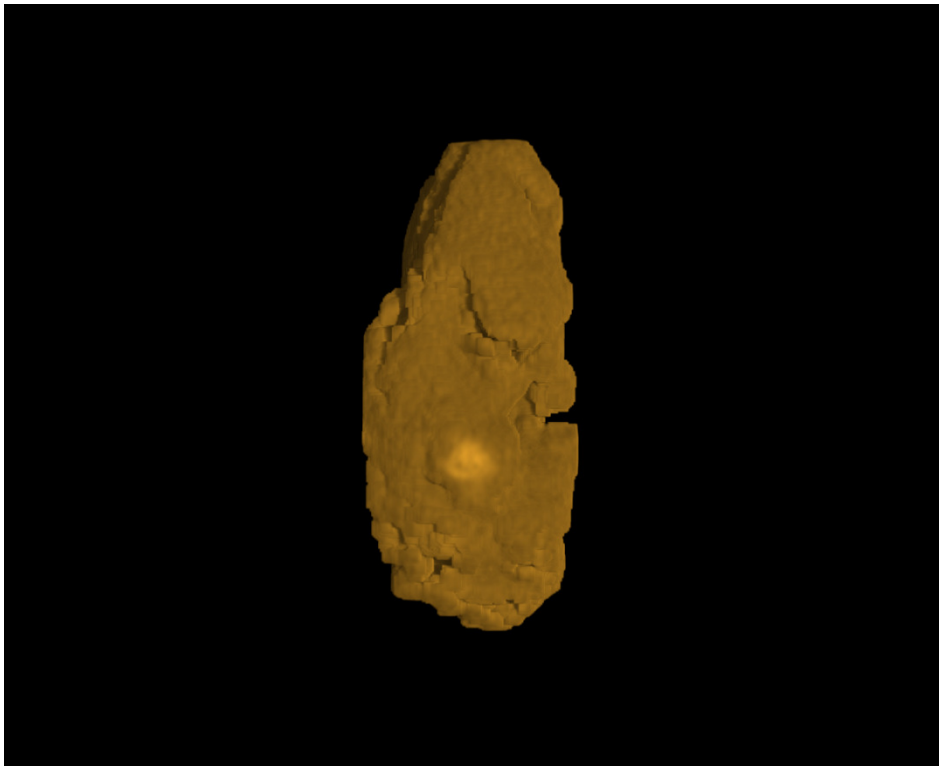


Figure 38 - (above) – FWD (Class III Day 21 - GLD100) - Superior view

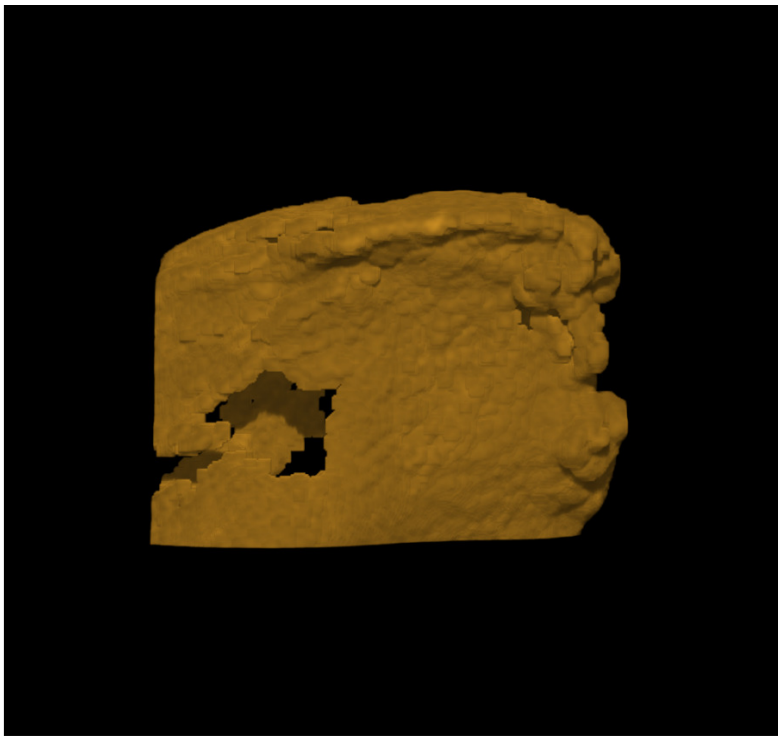


Figure 39- (above) – FWD (Class III Day 21 - GLD100) - Lateral view

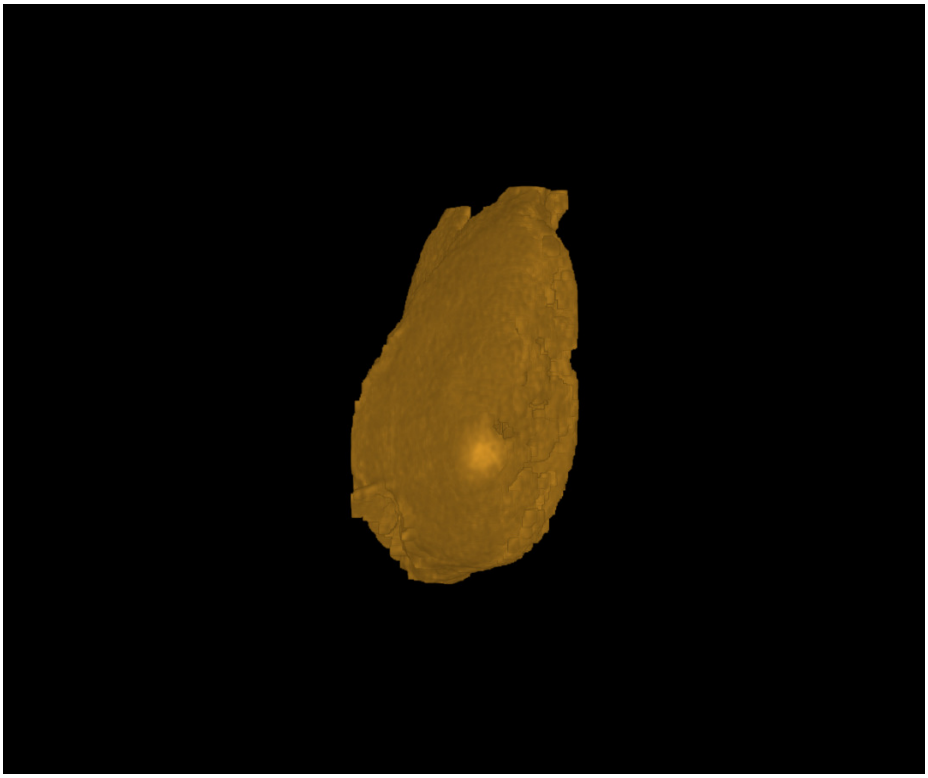


Figure 40 - (above) – BWD (Class II Day 28 – GLD099) - Superior view

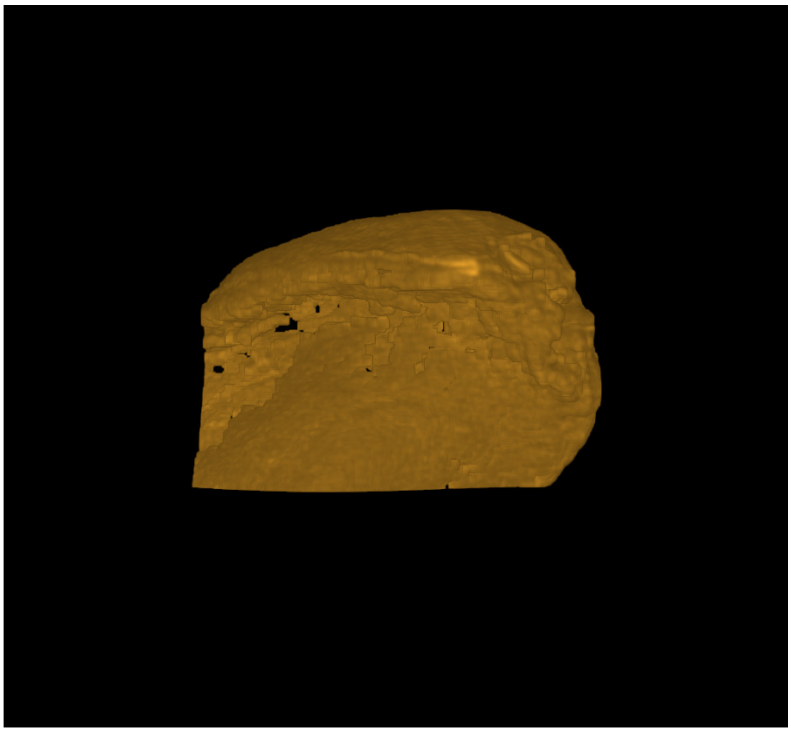


Figure 41 - (above) – BWD (Class II Day 28 – GLD099) - Lateral view

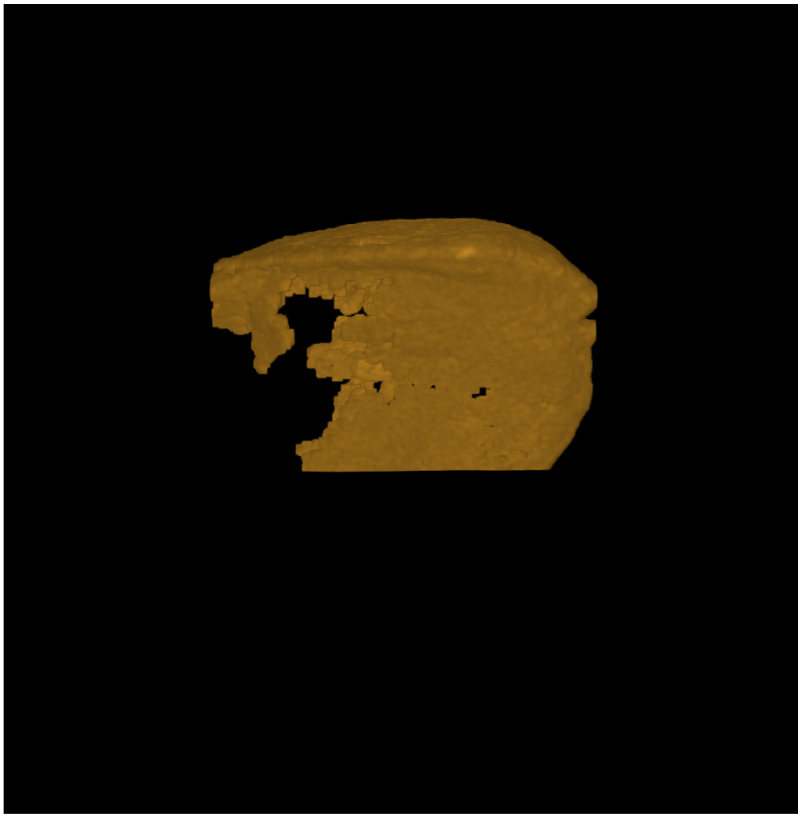


Figure 42- (above) - BWD (Class II Day 28 – GLD029) - Lateral view

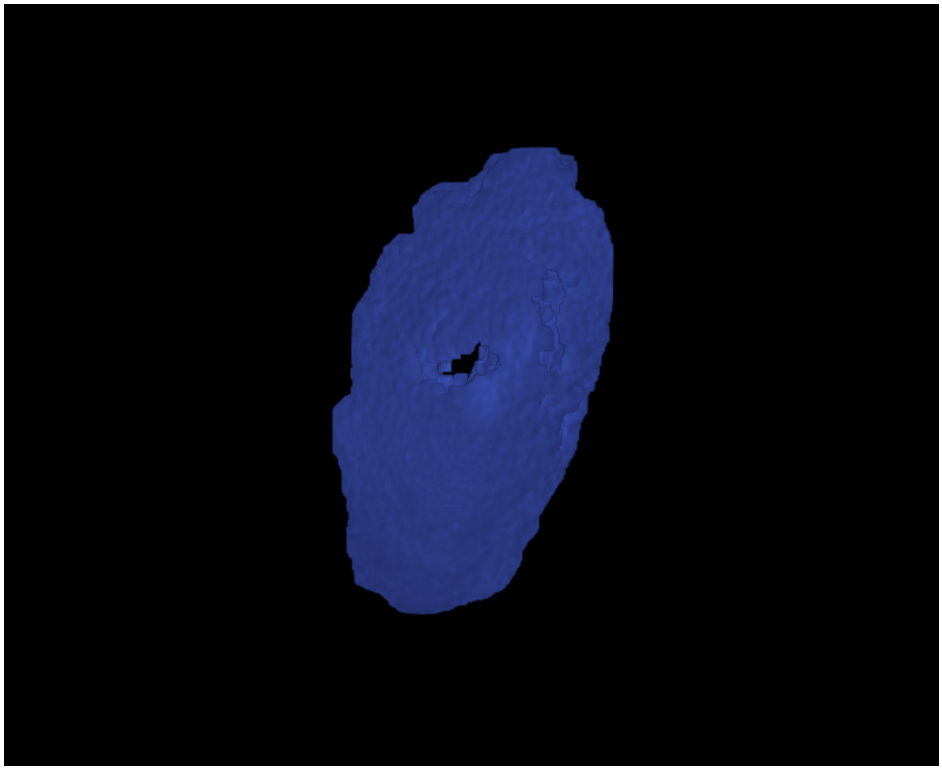


Figure 43 - (above) – FWD (Class III Day 28 – GLD026) - Superior view

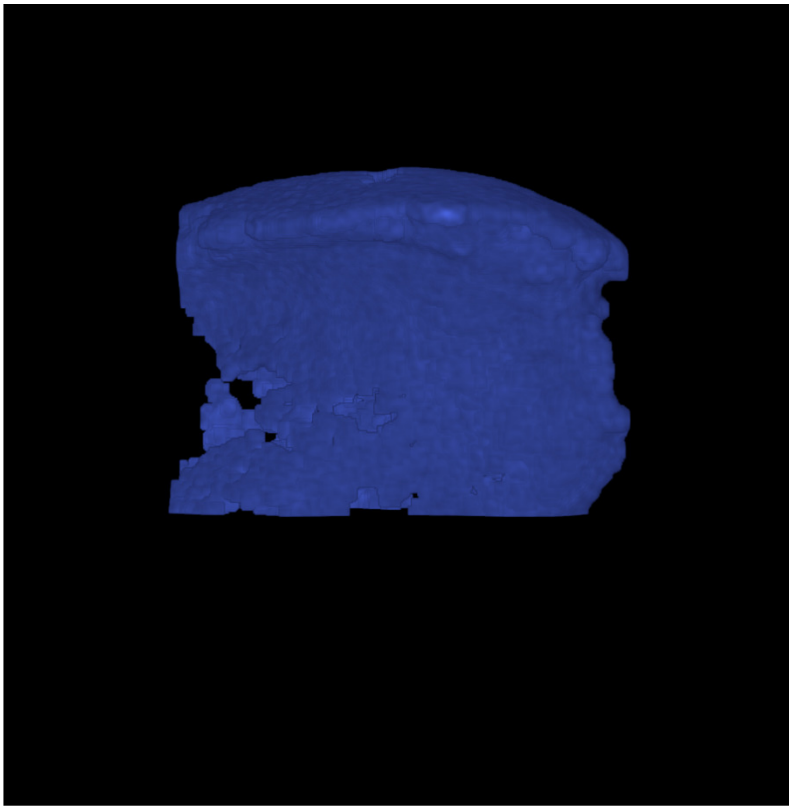


Figure 44 - (above) – FWD (Class III Day 28 – GLD026) - Lateral view

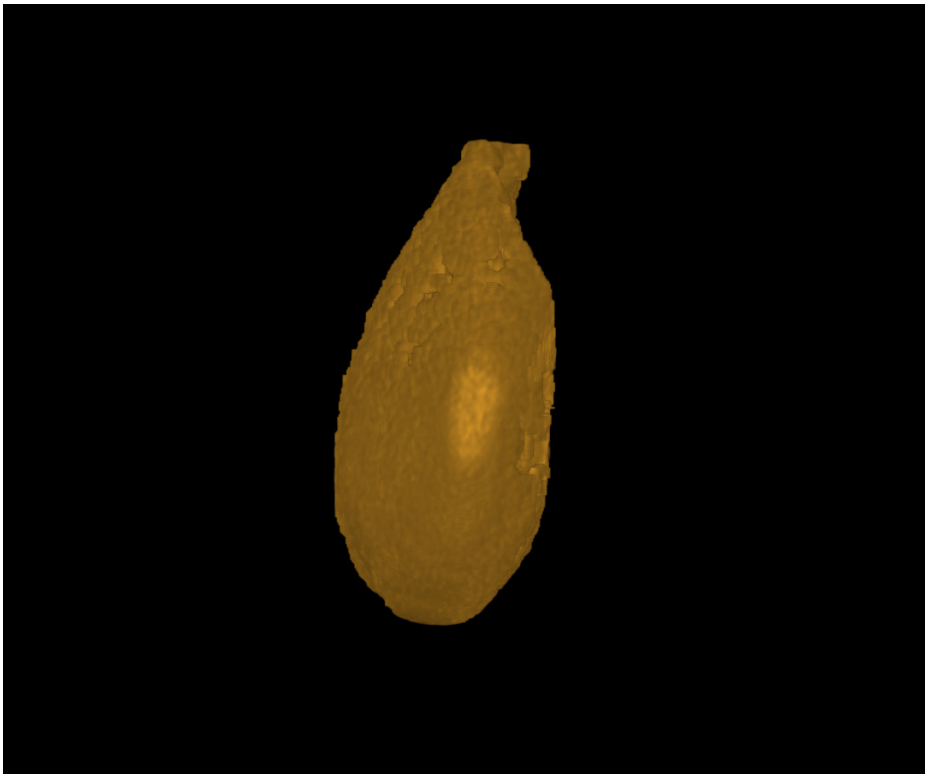


Figure 45 - (above) – FWD (Class III Day 7 – GLD030) - Superior view

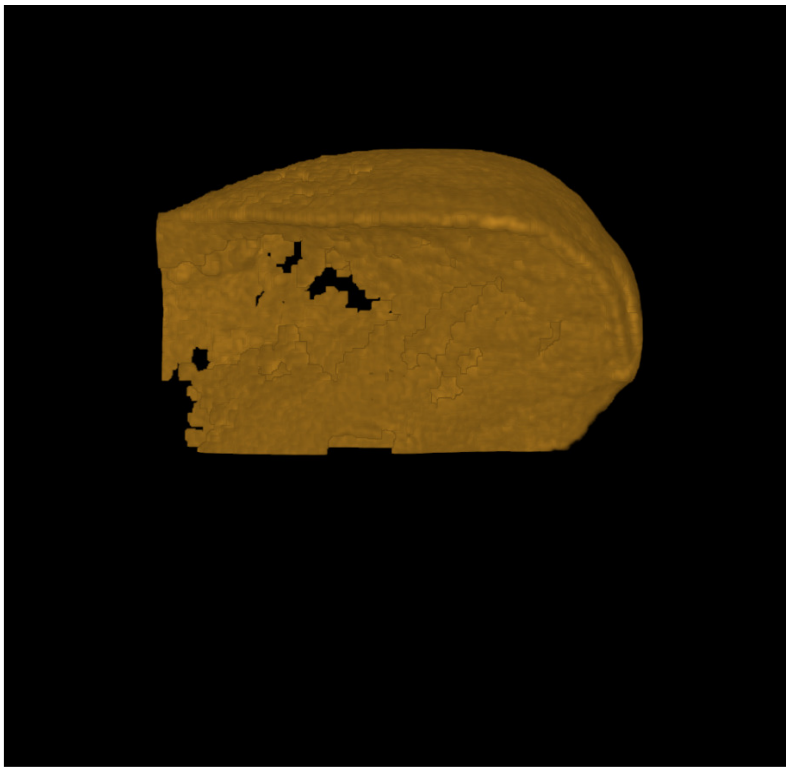


Figure 46 - (above) – FWD (Class III Day 7 – GLD030) - Lateral view

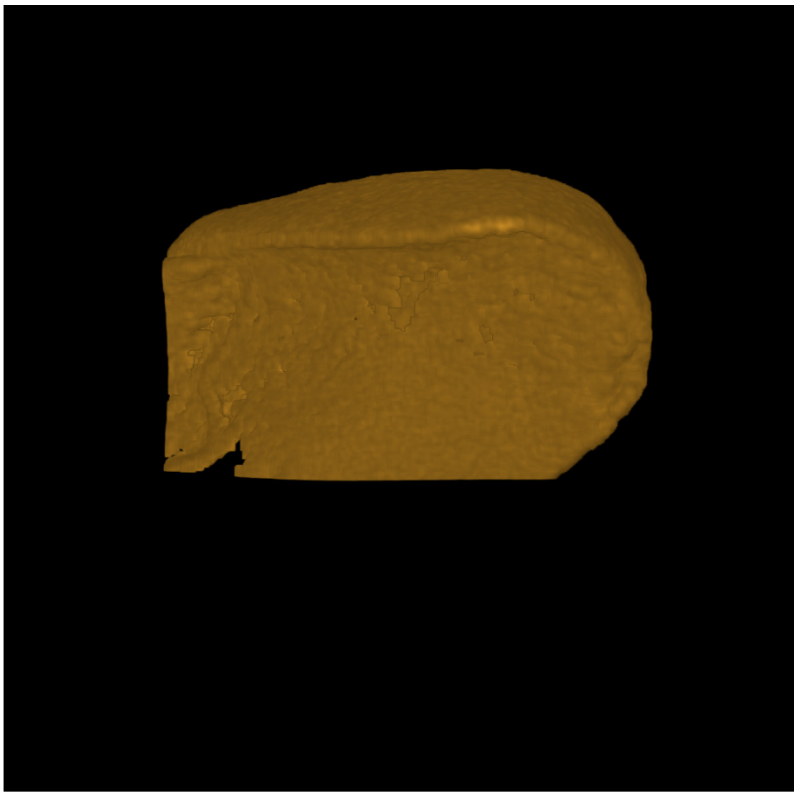


Figure 47 - (above) – FWD (Class III Day 7 – GLD091) - Lateral view

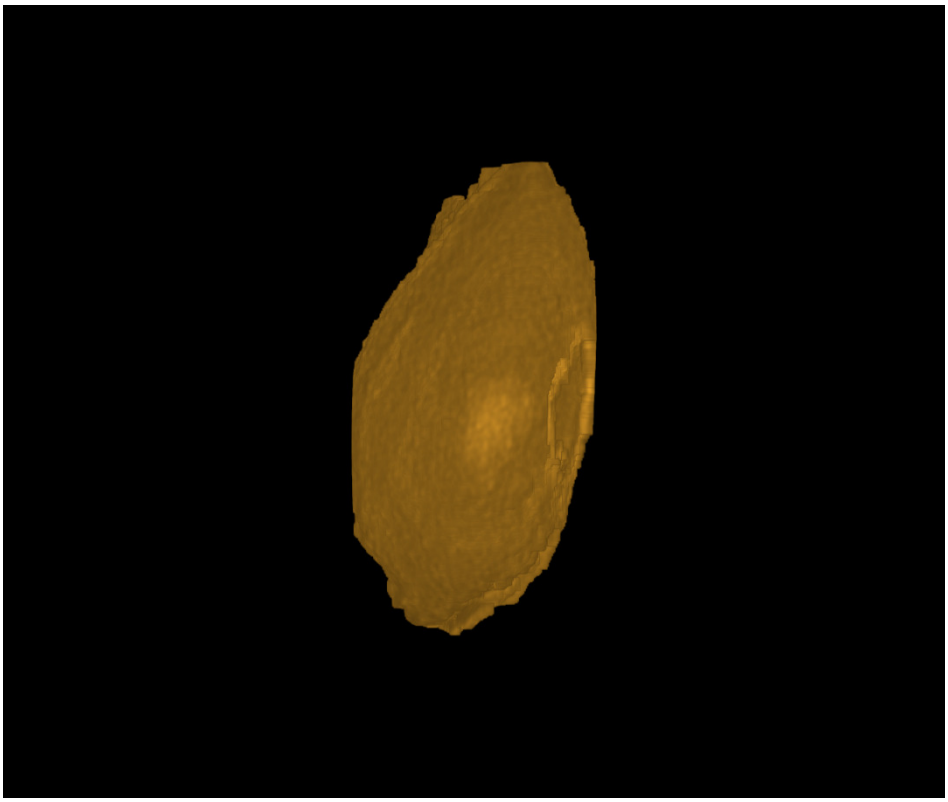


Figure 48 - (above) – FWD (Class III Day 7 – GLD104) - Superior

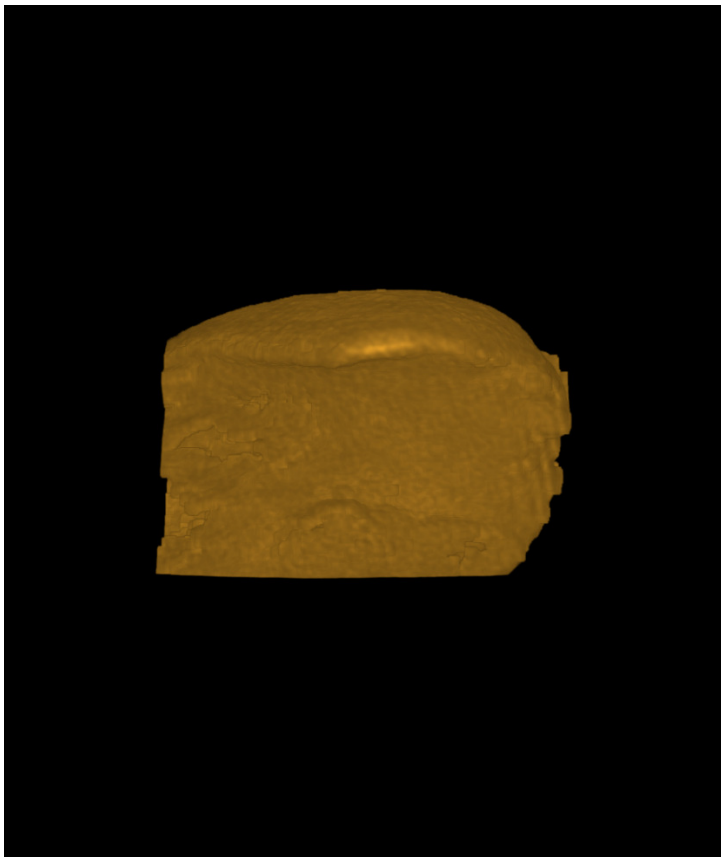


Figure 49 - (above) - FWD (Class III Day 7 – GLD104) - Lateral view

14 Tables

Name of Sample	GLD001R_6days_5um	Date of Analysis	29-01-2008

VGStudioMax – preorientated

Threshold (No gauss)	49-50					
Gross clip box	X (599)	282-881	Y (599)	231-830	Z (899)	0-899

Orientation – rotation steps

Rotations	Gross rotations		Geometric		Standardised	
	Z-10 Z-15, X+5, Y+5 (X-15)		Z-10 Z-15, X+5, Y+5		Z-25, X-10, Y+5	
RotateScanline adjustment	X (YZ)	+10	Y (XZ)	+5	Z (XY)	+25
Checked geometric centre box: superimposition required			Checked			

Data Analysis

Clip Boundaries of whole condyle	X (IS 361)	186-546	Y (LM 279)	172-450	Z (PA 562)	98-659
Clip Boundaries of anterior condyle	X (IS 361)	186-546	Y (LM 279)	172-450	Z (PA 281)	378-659

Volume of whole cartilage + bone	Gauss	1	Static	118	Exp	5	27110860
Volume of whole cartilage	Gauss		Static	100	Exp	4	8435795
Noise	Remove noise → moderate left						
Volume of ant cartilage + bone	Gauss	1	Static	122	Exp	5	12267711
Volume of ant cartilage + bone	Gauss		Static	111	Exp	4	4091725
Noise	Remove noise → minimal left						
Notes	(1) Hole in condyle – merged						

Remeasured	15-06-2008						
Volume of whole cartilage + bone	Gauss	1	Static	90	Exp	5	28280488
Volume of whole cartilage	Gauss		Static	79	Exp	4	8934683
Noise	Remove noise → moderate left						
Volume of ant cartilage + bone	Gauss	1	Static	109	Exp	5	13178887
Volume of ant cartilage + bone	Gauss		Static	107	Exp	4	4204185
Noise	Remove noise → moderate-minimal left						
Notes	(1) Moderate to severe noise (2) Shape appears normal – smooth appearance (3) Cartilage – hole (middle to supero-medial) – contact with ridge a. Lateral pick up (90-100%)/ Medial 100%						

Table 1 - Example of Data collection sheet formulated for this study - identifies parameters examined (note all measurements are in voxels or pixels)

Table 2- Univariate ANOVA of Volumetric changes

Dependent Variable (Average)	Day									Class						
	Estimated Marginal Means								P-value	Estimated Marginal Means						P-value
	0		7		21		28			0 (Control)		2 (BWD)		3 (FWD)		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE	
Total Volume (AvTvol mm^3)	2.813[a]	0.092	2.417[a]	0.056	2.335[a]	0.053	2.218[a]	0.053	0.000	2.372	0.039	2.539	0.072	2.427	0.60	0.124
Total Cartilage Volume (AvTcart mm^3)	1.152[b]	0.053	0.940[b]	0.032	0.875[b]	0.031	0.927[b]	0.031	0.000	0.900[c]	0.022	0.980	0.042	1.041[c]	0.35	0.004
Posterior Total volume (avTvolP mm^3)	1.424[d]	0.087	1.262	0.053	1.134[d]	0.050	1.145[d]	0.050	0.014	1.195	0.036	1.340	0.068	1.190	0.056	0.136
Posterior Cartilage volume (AvPcart mm^3)	0.623[e]	0.055	0.495[e]	0.034	0.438[e]	0.032	0.498[e]	0.032	0.037	0.442[f]	0.023	0.540	0.043	0.559[f]	0.036	0.015

Bonferroni Adjustment for Multiple comparisons notes:

[a] – There is a difference between Day 0 and 7, 21, 28 in Total volumes only (p-value <0.002)

[b] – There is a difference between Day 0 and 7, 21, 28 in Total Cartilage volumes only (p-value <0.001)

[c] – There is a difference between Class 0 and 3 in Total Cartilage volumes only (p-value <0.004)

[d]– There is a difference between Day 0 and 21, 28 in Posterior Total volumes only (p-value <0.033)

[e]– There is a difference between Day 0 and 7, 21, 28 in Posterior Cartilage volumes only (p-value <0.001)

[f] – There is a difference between Class 0 and 3 in Posterior Cartilage volumes only (p-value <0.001)

Table 3 - Method Error Analysis – T-Test

	Paired sample Correlations		Paired Differences				Error Analysis
Dependent Variable	**Correlation**	**P-Value**	**Mean**	**SD**	**SE**	**P-Value (2-tailed)**	**Co-efficient of variation**
Total Volume (Tvol)	0.951	0.000	0.008345	0.179576	0.022104	0.707	5.37%
Total Cartilage Volume (Tcart)	0.911	0.000	-0.017507	0.090388	0.011126	0.120	6.83%
Posterior Total Volume (TvolP)	0.934	0.000	0.008585	0.143247	0.017632	0.628	8.94%
Posterior Cartilage Volume (Pcart)	0.874	0.000	-0.019481	0.089769	0.011050	0.083	13.32%

Table 4 - Effects of Day Intervals on Classes – significant interactions

	AvTvol		AvTcart		AvTvolP		AvPcart		Weight at sacrifice	
	Interval[a]	**P-value[b]**	**Interval[a]**	**P-value[b]**	**Interval[a]**	**P-value[b]**	**Interval[a]**	**P-value[b]**	**Interval[a]**	**P-value**
Class 0	**Day 0-21** **Day 0-28**	**0.004** **0.001**	**Day 0-21** **Day 0-28**	**0.003** **0.043**		**ns**	**Day 0-21**	**0.020**		**ns**
Significance of Days		**0.001**		**0.005**		**ns**		**0.028**		**ns**
Class 2		**ns**		**ns**		**ns**		**ns**	**Day 0-7** **Day 0-21** **Day 0-28**	**0.039** **0.000** **0.016**
Significance of Days		**ns**		**ns**		**ns**		**ns**		**0.000**
Class 3		**ns**		**ns**		**ns**		**ns**	**Day 0-7** **Day 0-21** **Day 0-28**	**0.001** **0.000** **0.002**
Significance of Days		**ns**		**ns**		**ns**		**ns**		**0.000**

[a] - Bonferroni adjustment for multiple comparisons

[b] - Tcube covariant used

ns – not significant at the 5% confidence level

Table 5 - Effects of Day Intervals on Classes – significant interactions (cont.)

	ML		SI		AP	
	Interval	P-value	Interval	P-value	Interval	P-value
Class 0		ns		ns		ns
Significance of Days		ns		ns		0.05
Class 2		ns	Day 0-21 Day 7-21	0.002 0.011		ns
Significance of Days		ns		0.001		0.032
Class 3		ns		ns		ns
Significance of Days		ns		ns		ns

Table 6 - Effect of Class on Time points – significant interactions

	AvTvol		AvTcart		AvTvolP		AvPcart		Weight at sacrifice	
	Interval[a]	P-value[b]	Interval[a]	P-value[b]	Interval[a]	P-value[b]	Interval[a]	P-value[b]	Interval[a]	P-value
Day 7		ns		ns		ns		ns	Class 0 vs. 3	0.003
Significance of Class		ns		ns		ns		ns		0.003
Day 21	Class 0 vs. 2	0.010	Class 0 vs. 3	0.006	Class 0 vs. 2 Class 2 vs. 3	0.037 0.046	Class 0 vs. 2 Class 0 vs. 3	0.049 0.018	Class 0 vs. 2 Class 0 vs. 3	0.044 0.007
Significance of Class		0.012		0.004		0.026		0.007		0.004
Day 28		ns		ns		ns		ns	Class 0 vs. 3	0.012
Significance of Class		ns		ns		ns		ns		0.011

[a] - Bonferroni adjustment for multiple comparisons

[b] - Tcube covariant used

15 Appendix

15.1 NUTRIGEL

Illustration 1 – Example of Nutrigel gel (source: www.troylabs.com.au)

Distributor:
Troy Laboratories Pty. Ltd (www.troylab.com.au)

Incorporating ILIUM Veterinary Products

98 Long Street, Smithfield NSW 2164 Australia

PO Box 6626, Wetherill Park NSW 2164 Australia

Tel: (+612) 9604 6266 Fax: (+612) 9725 1772
Uses:
Nutrigel is described as a palatable high calorie, mineral/vitamised dietary supplement for use in dogs and cats lacking adequate nutrition over a prolonged period of time in the form of a highly palatable paste with protein, fats and carbohydrates. It helps to stimulate appetite while providing calories and other dietary essentials to debilitated or convalescent animals, or those recovering from surgery.

Composition:
Contains Vitamins A, D, E, B1, B2, B6, B12, Nicotinamide, Calcium Pantothenate, Folic Acid, Iron, Manganese, Magnesium and Iodine in a palatable base consisting of fats, proteins and carbohydrates, providing approximately 1500 kilojoules of metabolizable energy per 100g.

Actions:
Provides extra energy, vitamins and minerals. Appetite stimulant.
Indications A highly palatable source of calorie and vitamin supplementation and is recommended for inclusion in the diets of dogs and cats where levels may be low. Nutrigel can be used to overcome the animal's reluctance to the intake of tablets by fully covering the tablet with Nutrigel.

Dosage and Administration:
It is highly palatable and is usually accepted eagerly. To acquaint your pet with the flavour, place a small amount in the mouth. When used during convalescence and to stimulate appetite, give 10g (approx. 2 x 10cm strips) daily per 5kg bodyweight. When used as the main source of nutrition, give 15g (approx. 3 x 10cm strips) daily per 5kg bodyweight, or as required.

Presentation: Gel 200g.

Storage: Store below 30°C (room temperature).

15.2 Formulation for 200mM Gadolinium Chloride

The Molecular Weight of $GdCl_3$ = 263.61g
Therefore 1 mol of GdCl3 =263.61g/1000ml of liquid
Hence the amount of $GdCl_3$ in 25ml = 263.61/40 = 6.59g
Hence for 25ml of liquid, to make 1mol, 6.59g of GdCl3 is required
To make 200mmol (20% of 1mol), 6.59g/5 = 1.318g

Thus for 200mmol of $GdCl_3$ = 1.318g/25ml

15.3 SkyScan 1172 Settings

Acquistion Mode	Ctrl+Shift+Alt+S
Pixel size	Medium (2000x1048x1048)
Filter	No
Resolution	5um
Step size	0.39um
KV	60Kv
Ma	161um
To set saturation/acquire flat field	Acquisition mode → marked mode (near medium 590*) → acquire flat field for marked mode

Settings for acquisition mode

		Filter 0
Far	Small	2945
	Medium	885
	Large	632
Medium	Small	1767
	Medium	147
	Large	77
Near	Small	589
	Medium	590*
	Large	79

Acquiring Flat field

(1) Narrow resolution down to 7um
(2) Make sure medium pixel size
(3) Set Acquisition mode settings and asterix near medium (arrow should be on near medium)
(4) Wait 5-10mins for beam to warm up before flat field
(5) Check asterix and arrow
(6) Flat field

15.4VGStudioMax – Imaging protocol

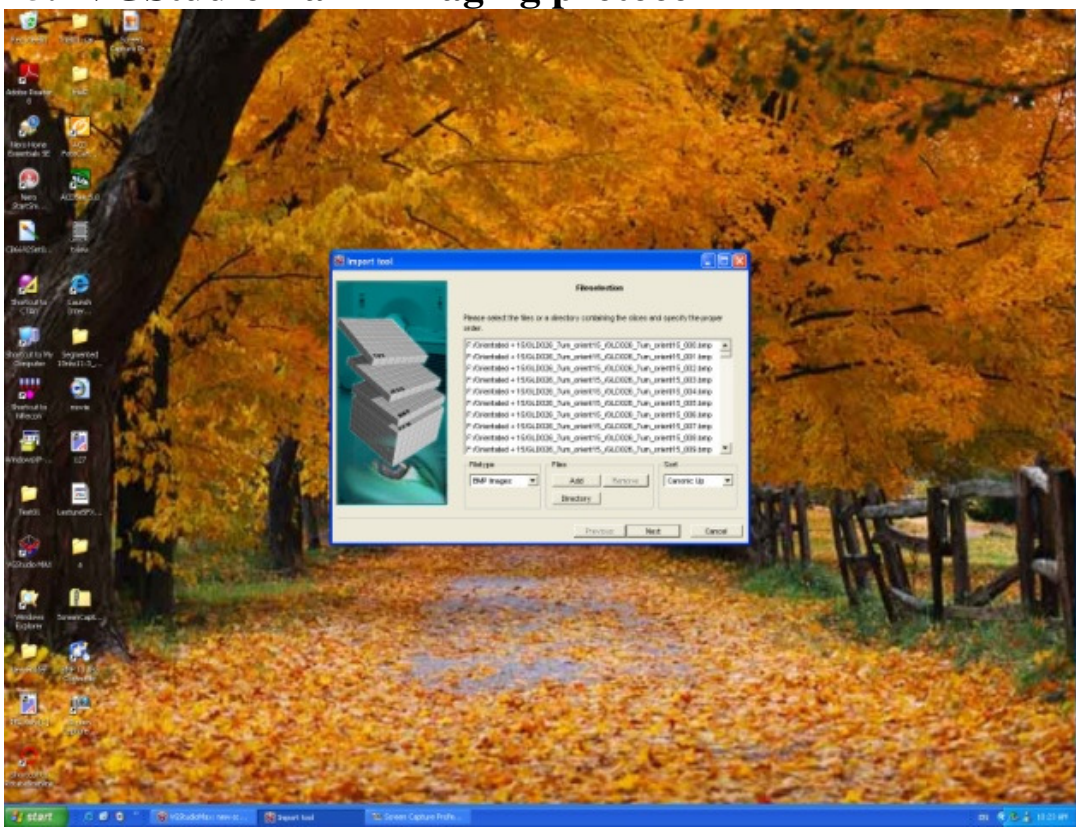


Illustration 2 – Once VGStudio Max is loaded, import image stack via Import Tool

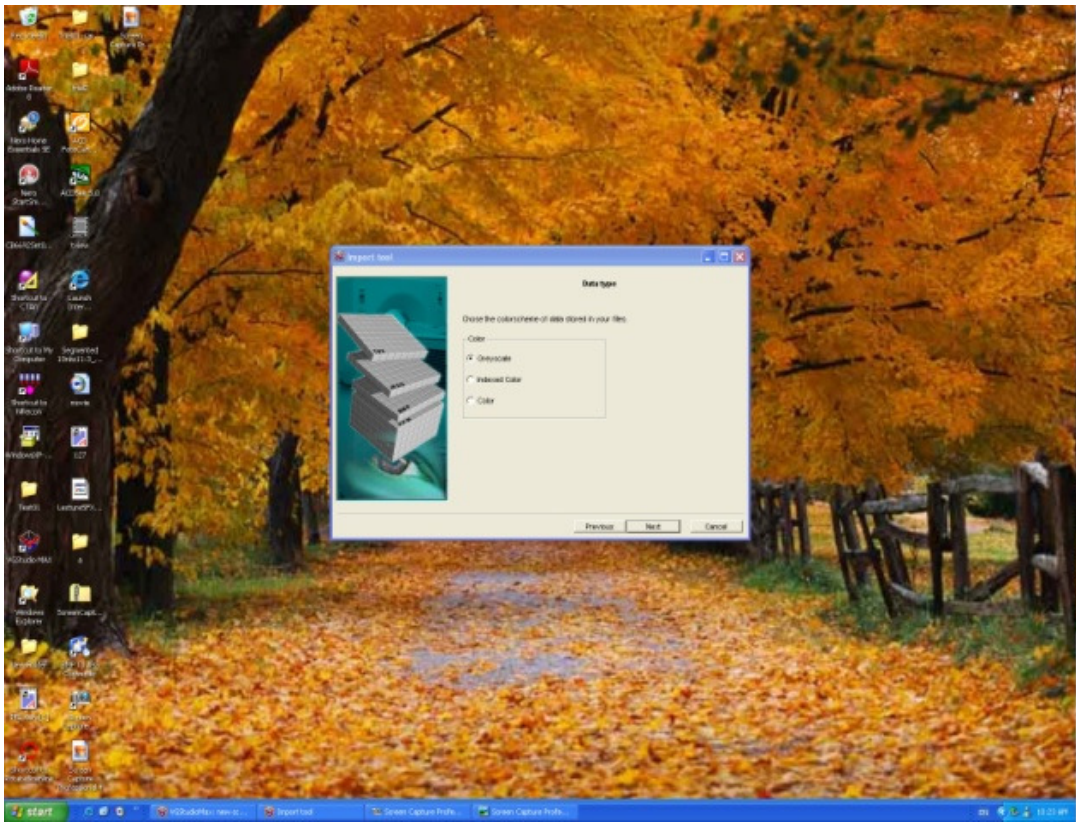


Illustration 3 - Images are imported as grayscale images

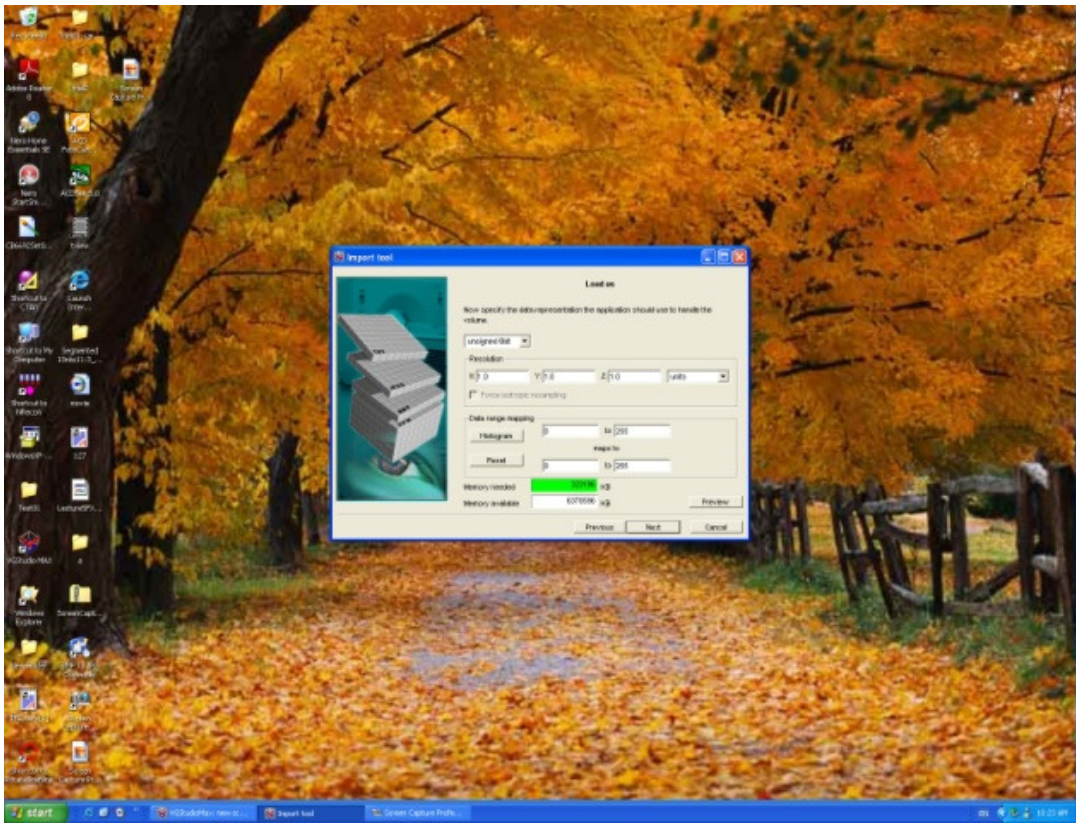


Illustration 4 – The Preview tool was used to isolate the reconstructed data to a maximally predetermined box dimension of 600x600x900 pixels that contained the region of interest

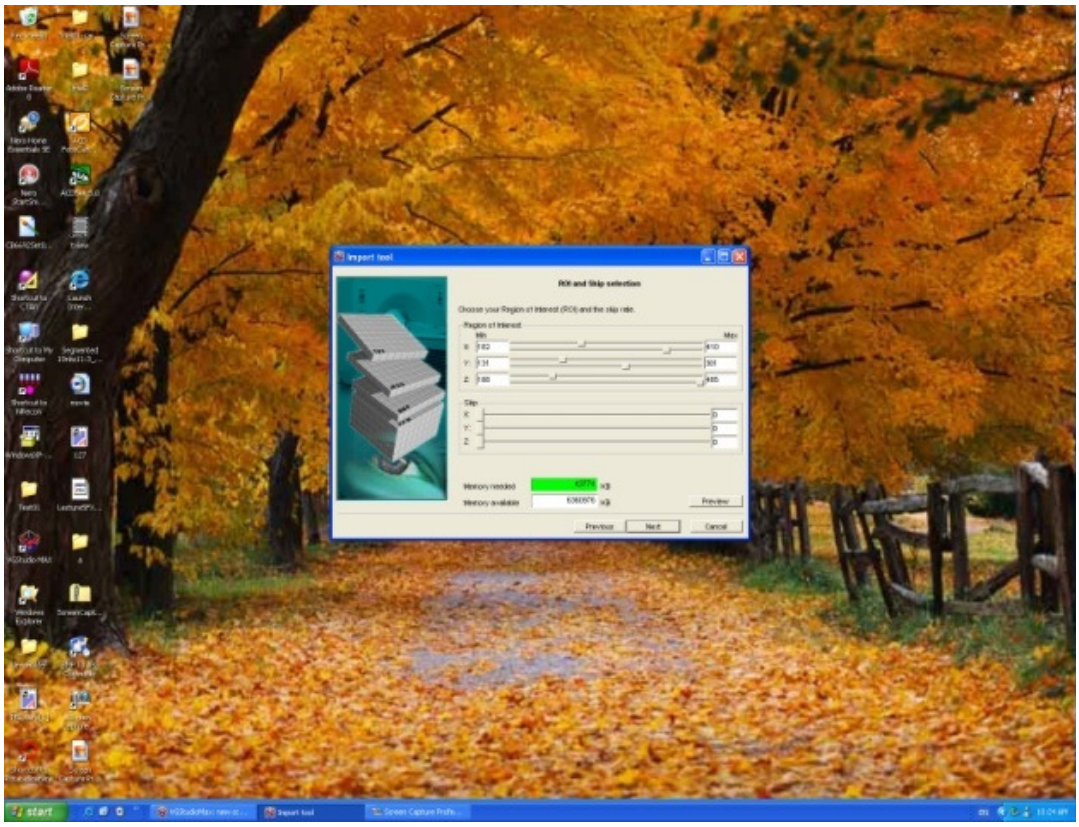


Illustration 5 - The Region of Interest tool helps restrict the data to only the area of interest when assessing the data initially prior to orientation and later during image analysis

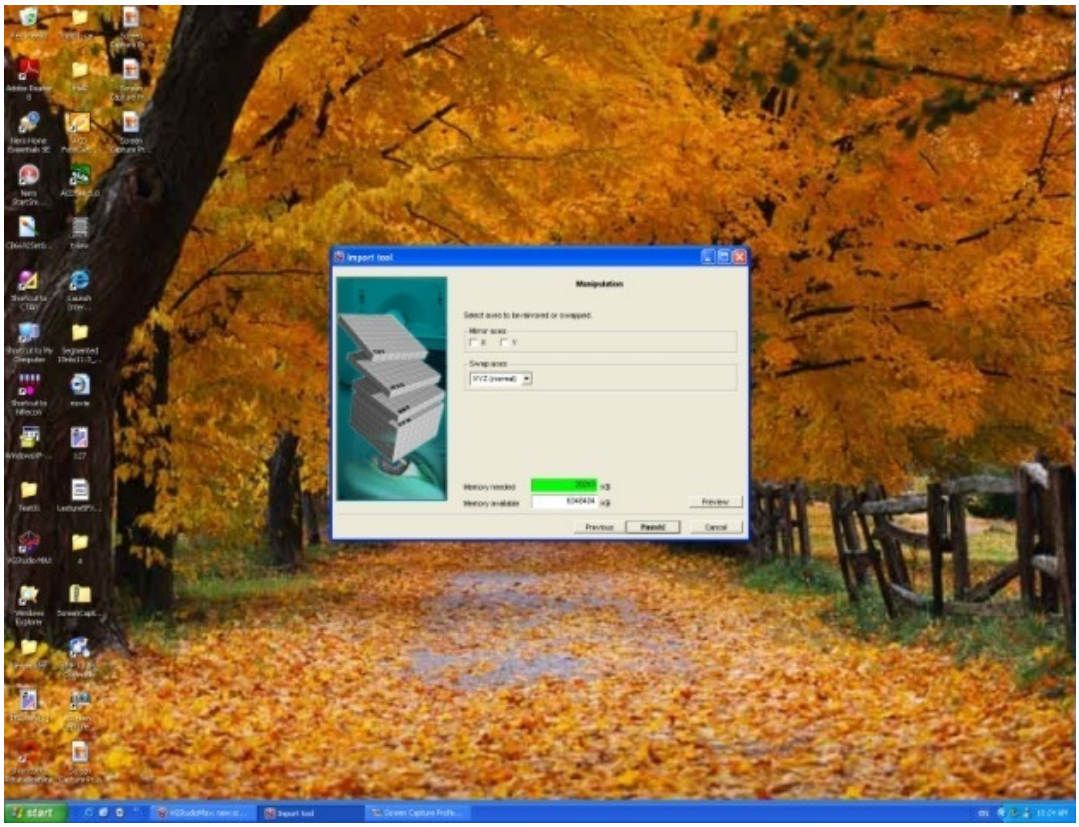


Illustration 6 - No manipulation was carried out of the raw data stacks

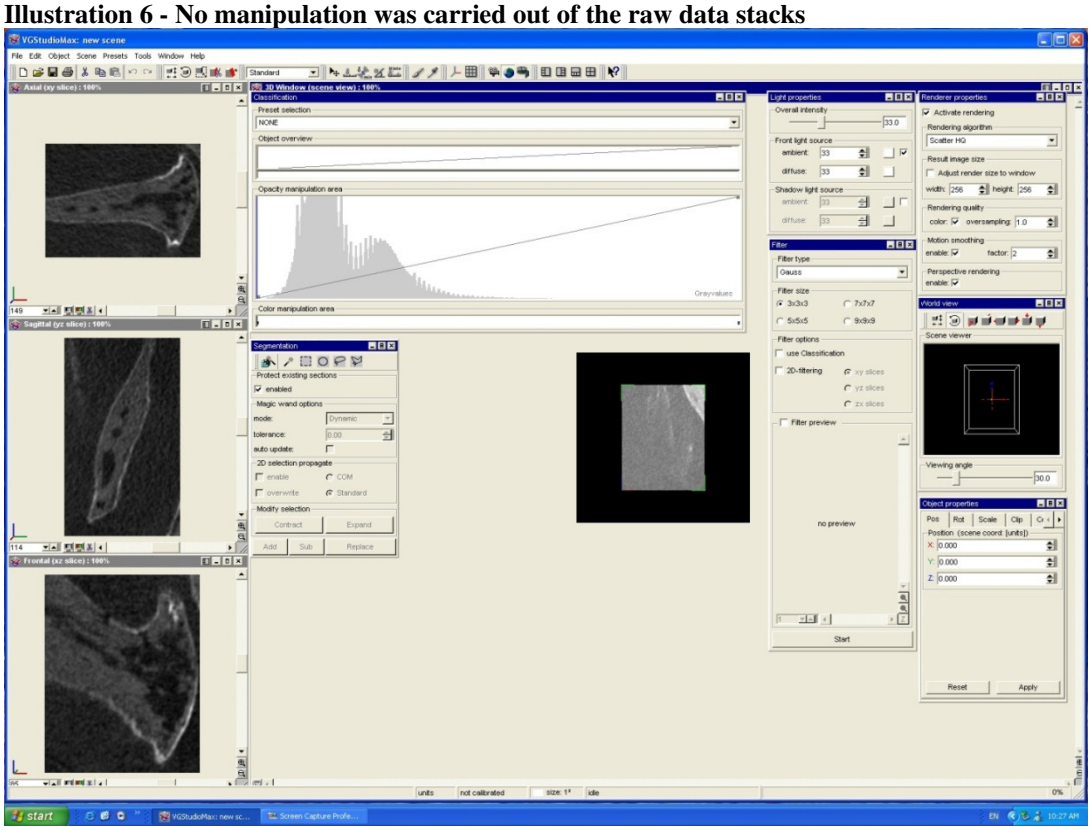


Illustration 7 - VGStudioMax after the importation of the reconstructed data displaying the necessary tools required for image analysis

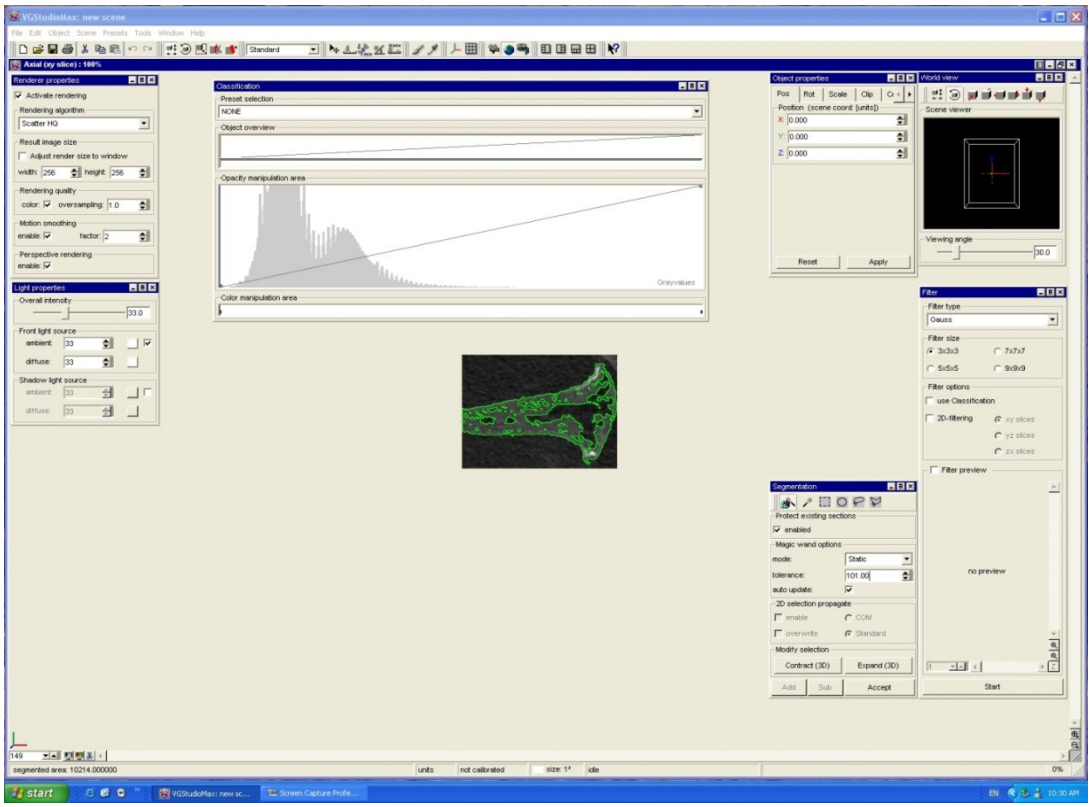


Illustration 8 – Depiction of the segmentation tool used to select an appropriate grayscale value to segment bone and cartilage from the surrounding noise (prior to expansion of the selection)

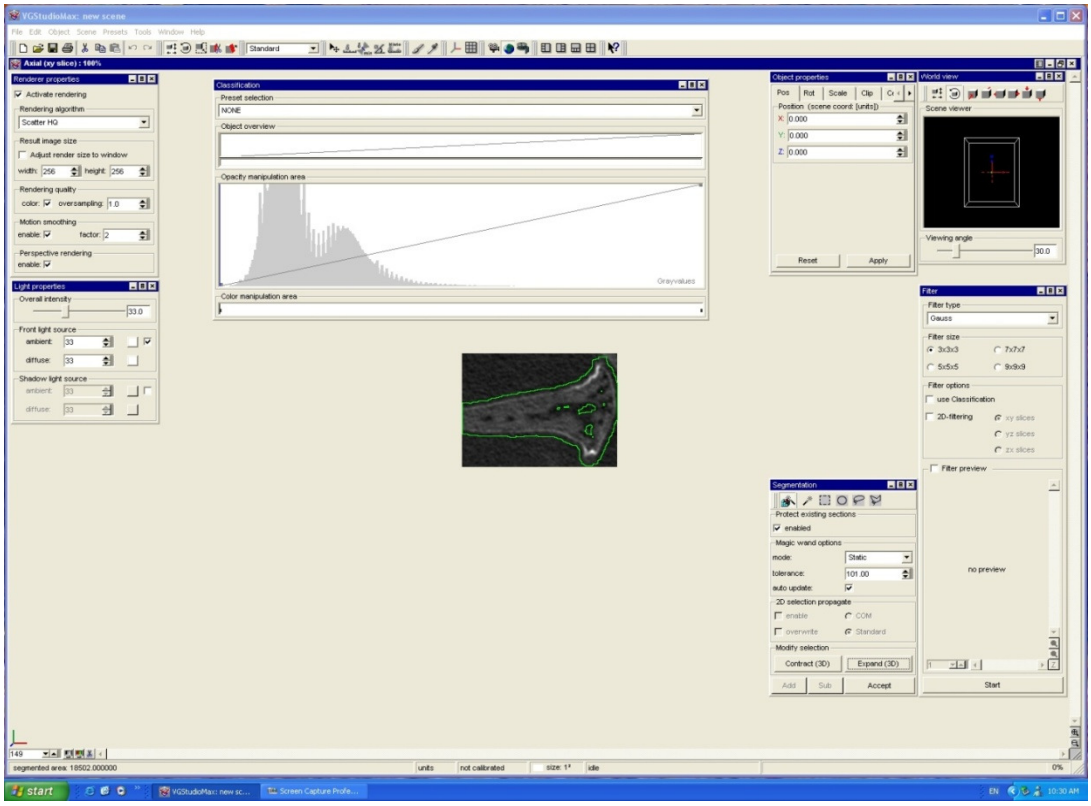


Illustration 9 - The selection was then expanded four times to ensure total selection of the condylar head. Note the difference in area selection compared to the previous Illustration

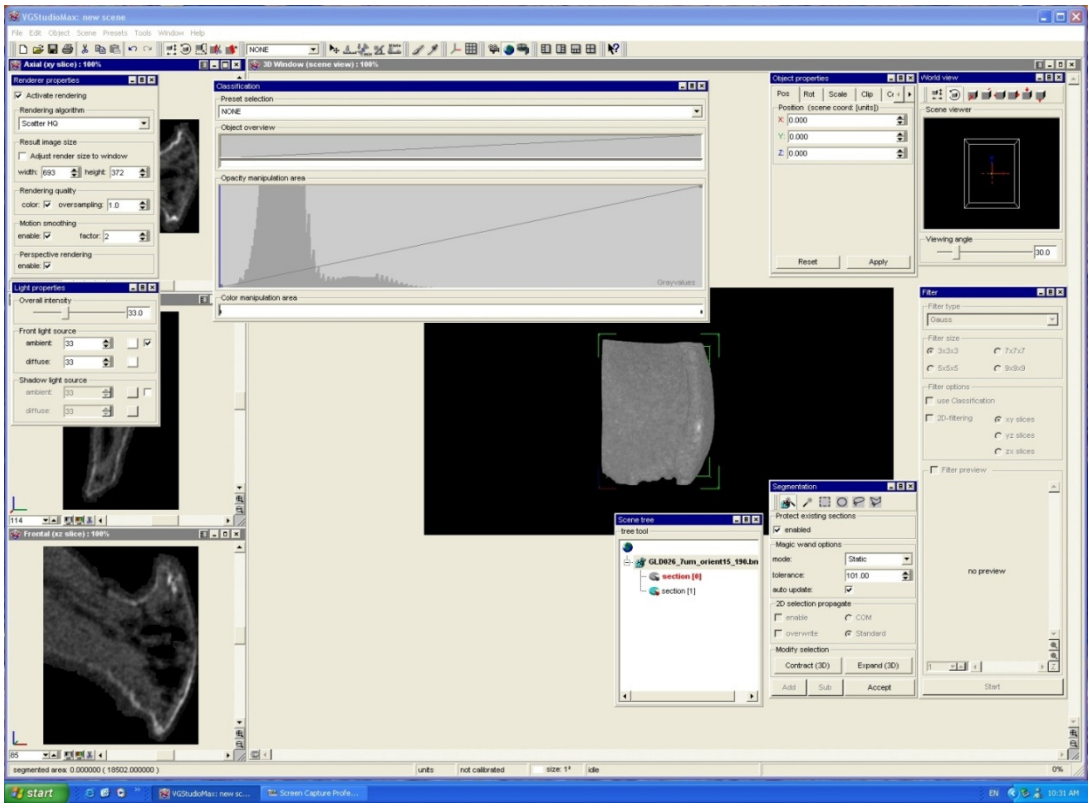


Illustration 10 - Three dimensional reconstruction of the total volume of the whole condylar head

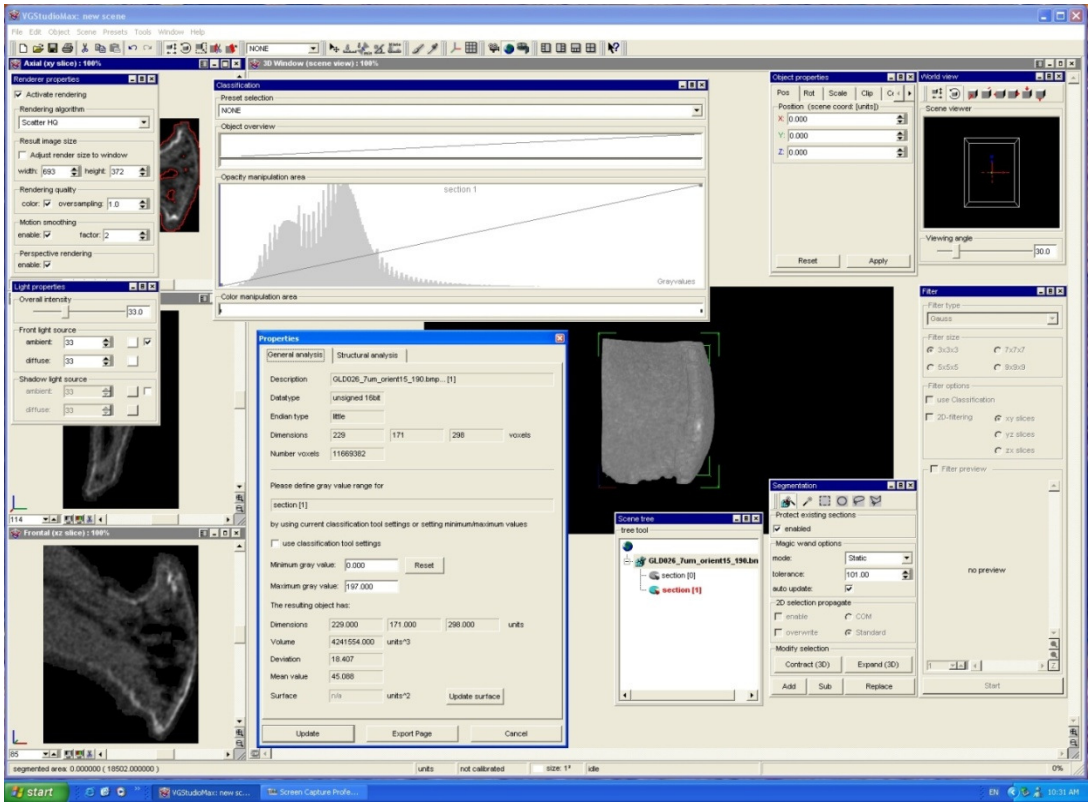


Illustration 11 - Volumetric measurements are tabulated by VGStudioMax giving the maximum linear dimensions ("Dimensions" - in pixels) and Volume (in voxels)

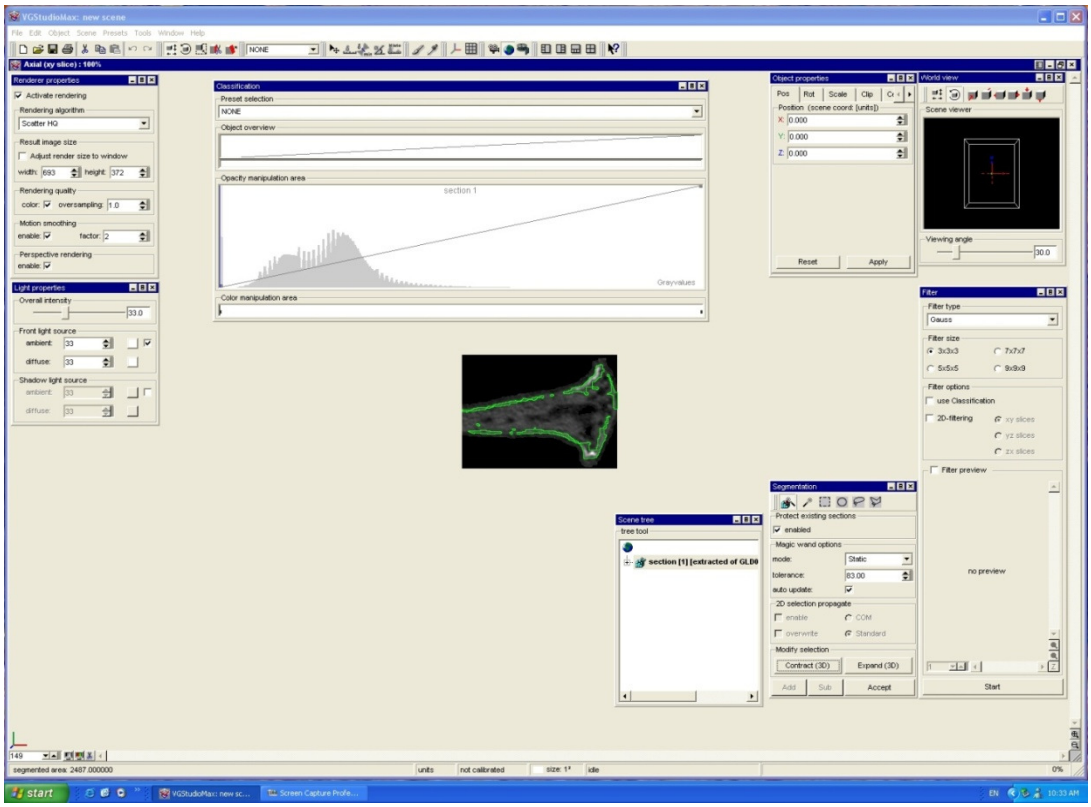


Illustration 12 - Segmentation tool is used to select the cartilage/periosteum

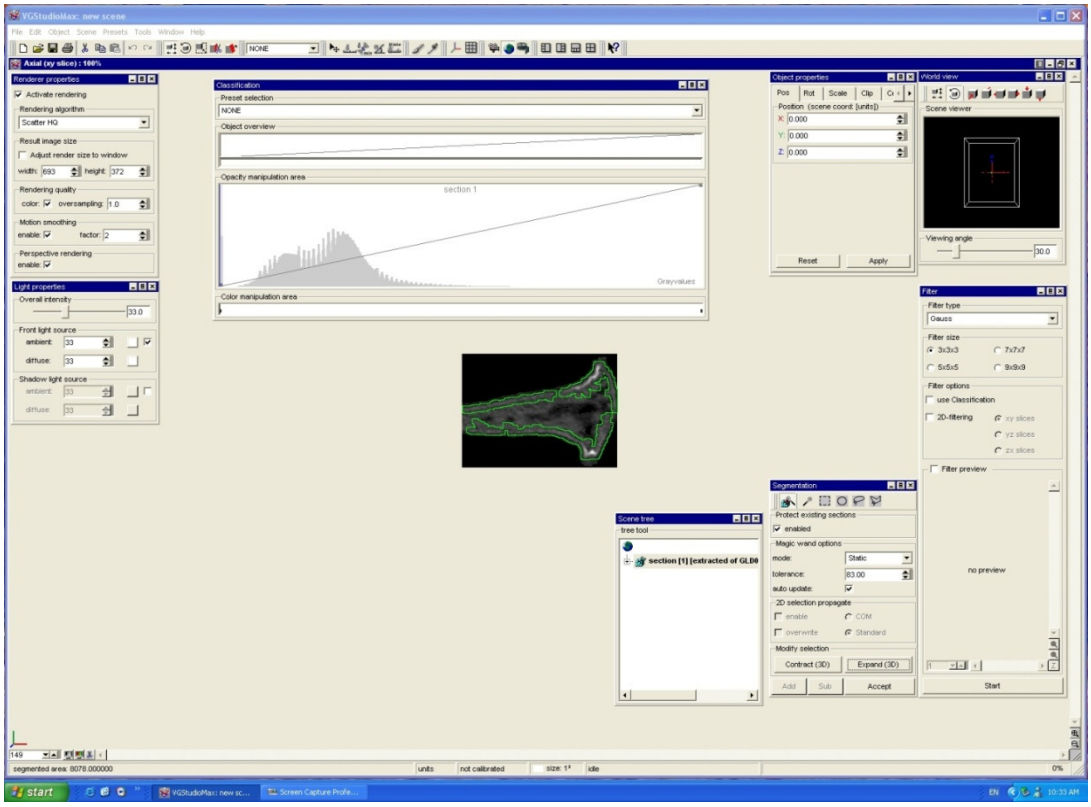


Illustration 13 - The cartilage/periosteum selection is then expanded three times. Note the difference in area selected from the previous Illustration

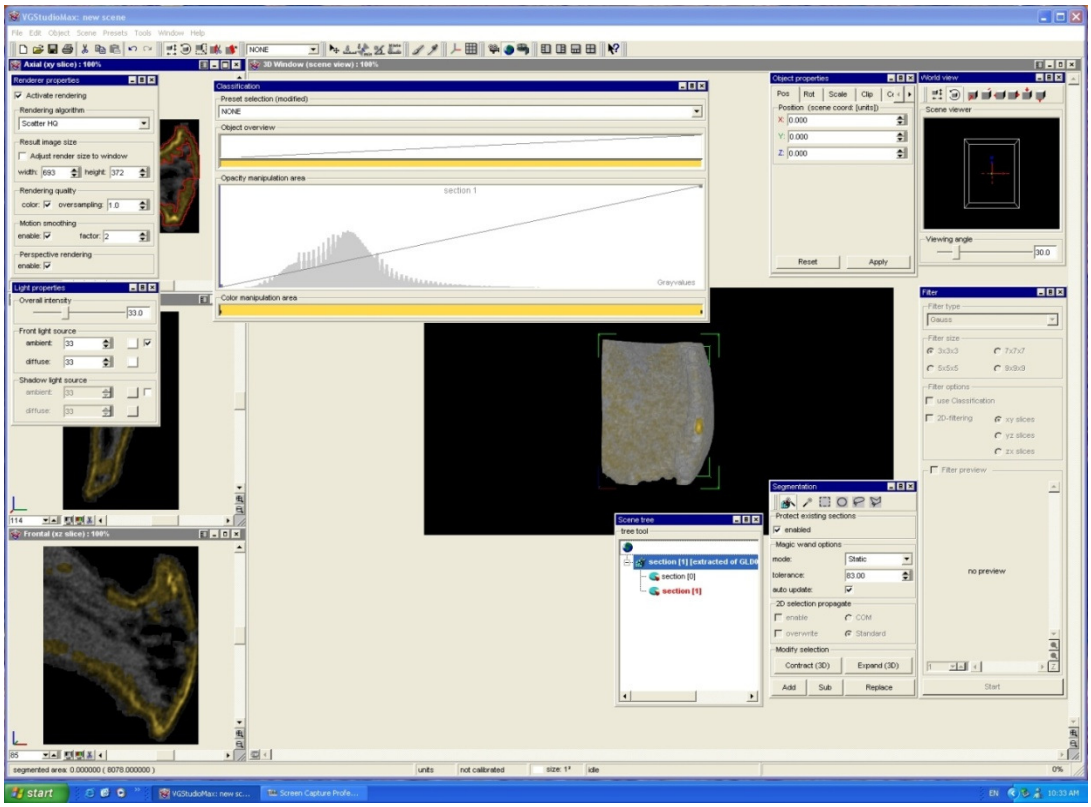


Illustration 14 - Depiction of the whole condylar head with the selected periosteum/cartilage highlighted in gold

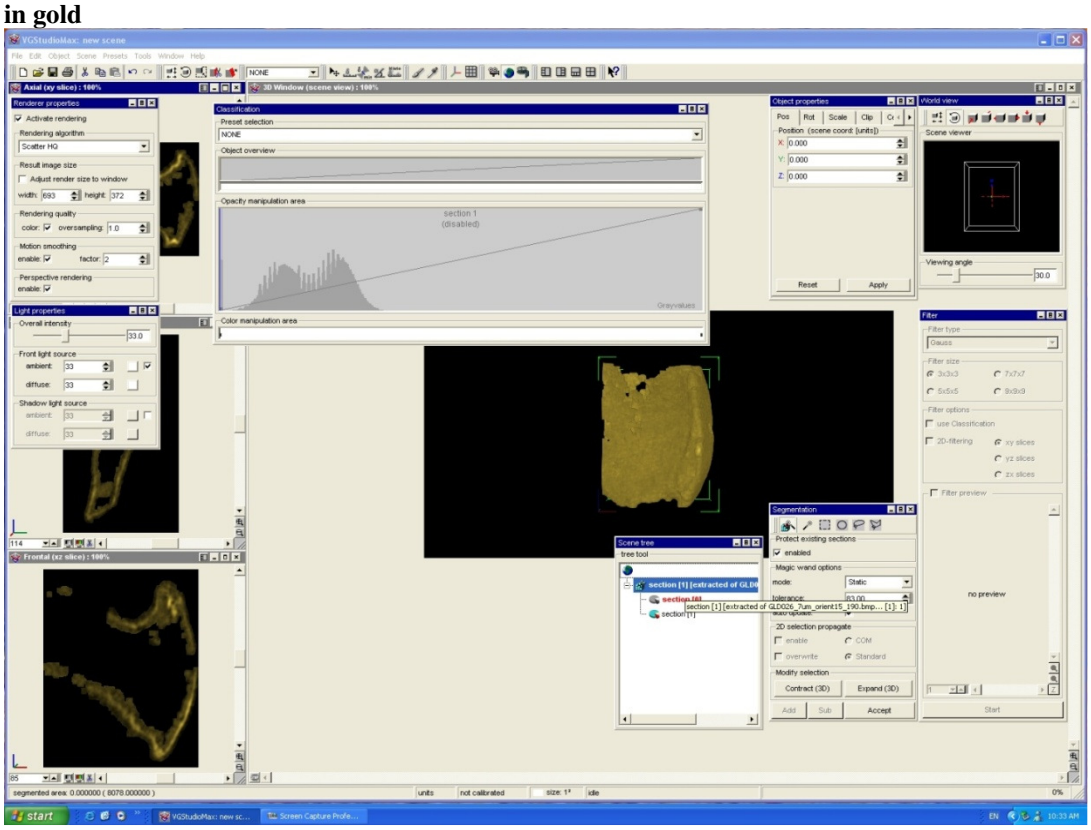


Illustration 15 - Demonstration of the total cartilage volume

15.5 RotateScanline

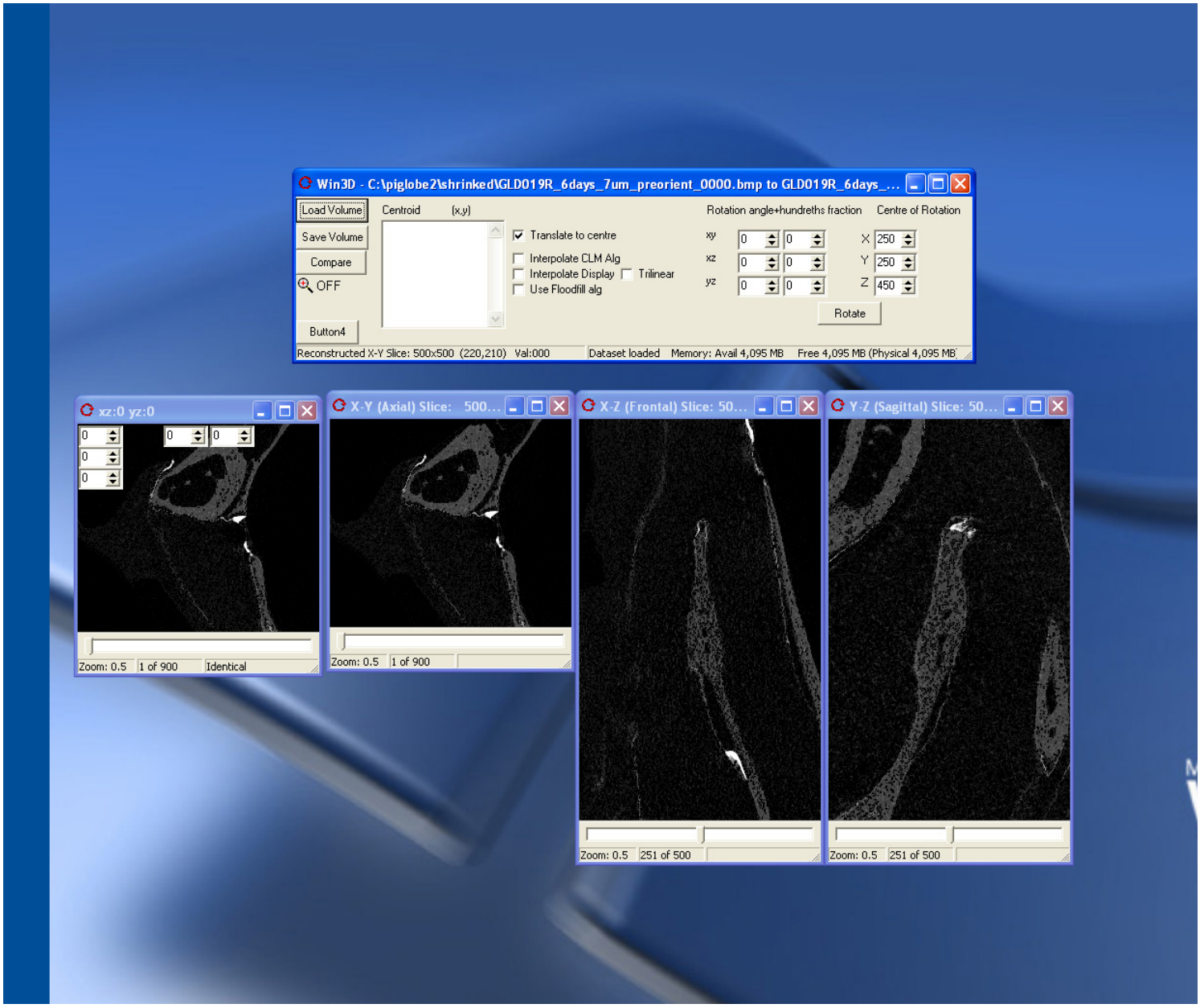


Illustration 16 - operating interface for RotateScanline

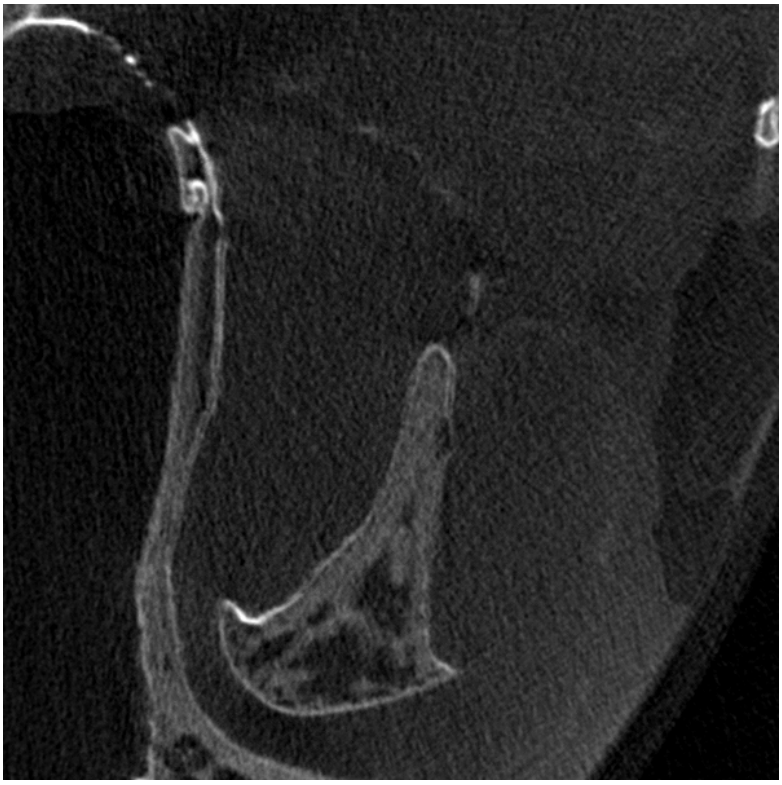


Illustration 17 - An axial slice of a preorientated condyle

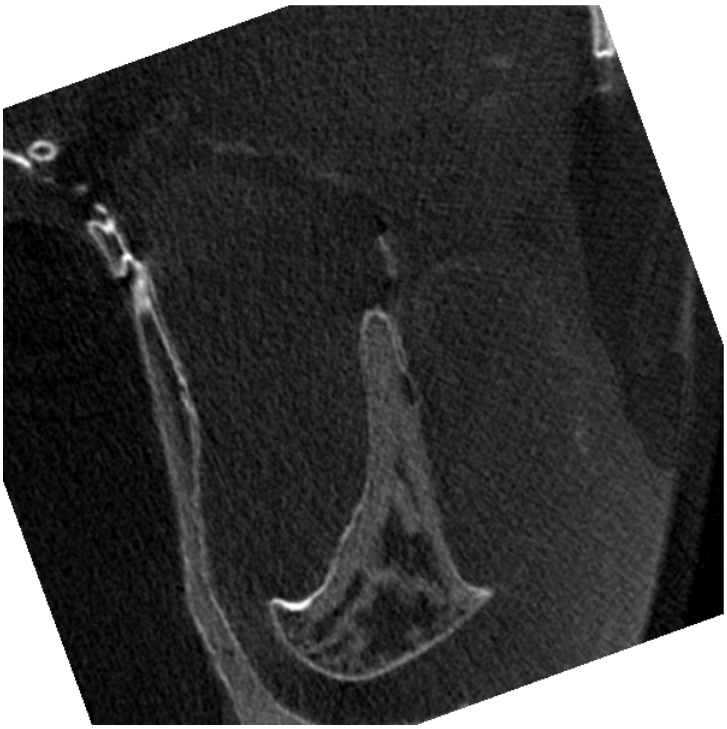


Illustration 18 - An orientated slice of the same specimen. Note the effects of rotation on the corners of the image

15.6 Calibrated Statistical Data

Sample	Day	Class	Total vol (mm3)	Re Total vol (mm3)	Cart Vol (mm3)	Re Cart Vol (mm3)
2	0	0	3.334987628	3.708380515	1.510062302	1.492165591
3	0	0	4.729561487	3.773992985	1.631039088	1.278569544
5	0	0	3.092695515	2.971442271	1.28157834	1.281359163
6	7	0	2.052367254	1.92423686	0.661172631	0.661172631
7	7	0	2.70480882	2.637079354	1.081958857	1.081958857
8	7	0	2.679431622	2.647992928	0.684582381	0.728393771
11	30	0	2.391396	2.391396	0.999074279	0.999074279
12	30	0	2.436493983	2.614301753	0.969532375	1.018397527
13	21	0	1.872714487	1.87356204	0.697346097	0.821364607
14	21	0	2.457392973	2.408784385	0.677684651	0.791518805
15	21	0	2.463789923	2.463789923	0.655241818	0.655241818
16	21	0	2.61079938	2.521484238	0.607661201	0.607661201
17	21	0	2.06505208	2.06505208	0.809277973	0.809277973
18	30	0	1.813135387	1.753415657	0.743761886	0.743761886
19	30	0	1.755351549	1.898267301	0.769764373	0.798147966
20	30	0	2.365034392	2.484070827	0.50816136	0.571299085
21	7	2	1.092376796	1.171021208	0.476820764	0.477198407
23	7	2	2.656587822	2.656588508	0.993286154	1.049388263
24	7	2	2.503511724	2.434662706	0.830490122	0.938142044
25	30	2	2.766778973	2.885379455	1.059312625	1.191257865
26	30	3	1.47200508	1.454853022	0.68053258	0.76639234
27	21	3	2.638670188	2.638670188	1.093972775	1.162886277
28	30	3	2.65428149	2.598230145	1.026490955	1.026490955
29	30	2	1.890673281	1.900852492	0.676316767	0.727604185
30	7	3	2.245892313	2.046027928	0.811804511	0.811106849
31	30	2	2.64973674	2.64973674	1.036639639	1.065057532
32	21	3	1.595818476	1.595818476	0.781856152	0.740694094
33	21	2	2.44139168	2.680058626	0.830644129	1.107081892
34	21	3	2.672665261	2.743537979	1.167094544	1.132378828
35	21	2	2.865999955	2.865999955	1.011757733	1.107081892
71	0	0	2.268610918	2.620886667	0.916463758	0.698580211
72	0	0	2.758406343	2.758418691	1.229622758	1.259654466
73	0	0	2.439224606	2.05323573	1.015350315	1.015350315
74	0	0	3.418419634	3.217271743	1.255141615	1.229533921
75	0	0	3.045762482	3.047376297	1.091646549	1.091647235
76	7	0	3.299778678	3.177320818	1.283055298	1.283055298
77	7	0	2.235772784	2.235772784	1.037793148	1.037793148
78	7	0	2.142252119	2.142252119	1.013561913	1.013561913
79	7	0	2.355634477	2.355634477	0.76226262	0.76226262
80	7	0	2.470448239		0.752938165	
81	21	0	2.30097091	2.212287231	0.87230045	0.750361892
82	21	0	1.756076994	1.746236324	0.847531734	0.847531734
83	21	0	2.021707856	2.021707856	0.771245104	0.771245104
84	30	0	2.05937886	2.05937886	0.955602459	0.955602459
85	30	0	1.793383046	1.906307564	0.736217601	0.736217601
86	30	0	1.965066894	1.896968703	0.912250689	0.912250689
87	21	0	1.795816631	1.774756774	0.703991379	0.616082194
88	21	0	2.123902305	2.129176616	0.842369241	0.924476924

Sample	Day	Class	Total vol (mm3)	Re Total vol (mm3)	Cart Vol (mm3)	Re Cart Vol (mm3)
89	30	0	1.787908766	2.167767546	0.840683396	0.840641893
90	30	0	2.087258586	2.087258586	0.904222774	0.904222774
91	7	3	2.155656216	2.147064409	1.007420841	0.949993723
92	7	3	2.88670172	3.003677411	1.028933458	0.93923347
93	30	3	1.550500287	1.64215023	0.702460227	0.702460227
94	30	2	2.938272456	2.938272456	1.208993366	1.208993366
95	7	3	2.569963858	2.367280013	1.068488218	1.0963309
96	30	3	2.230556783	2.221546173	0.996460619	0.986209378
97	30	3	1.601045796	1.602577291	0.909614734	0.942588696
98	7	3	2.061617621	2.186302237	0.923215027	1.13064119
99	30	2	2.608307828	2.516549154	1.129388211	1.052719136
100	21	3	1.743390796	1.655725484	0.712877137	0.712877137
101	21	3	2.389465253	2.310872291	0.944683397	0.942569488
102	21	3	1.418633594	1.508518459	0.675855432	0.675855432
103	21	2	2.537823043	2.785906711	0.829276245	0.909143109
104	7	3	2.403807798	2.102877091	1.022899059	1.038317595
105	21	2	2.558613302	2.639734174	0.803502882	0.948583993
1	0	0	3.3888575	3.535061	1.054474375	1.116835375
22	7	3	2.050761125	1.93709175	0.809538625	1.0941925

Note: GLD004, 009 and 010 Not Included in study due to issues with imaging process

Sample	Day	Class	Total ant (mm3)	ReTotalant (mm3)	Total post (mm3)	Re Total post (mm3)
2	0	0	1.808593038	1.704748416	1.52639459	2.003632099
3	0	0	1.917764107	1.431916269	2.81179738	2.342076716
5	0	0	1.30012778	1.207135678	1.792567735	1.764306593
6	7	0	1.081243702	1.074164182	0.971123552	0.850072678
7	7	0	1.405201714	1.304397787	1.299607106	1.332681567
8	7	0	1.106153734	1.106153734	1.573277888	1.541839194
11	30	0	1.2178558	1.172969448	1.1735402	1.218426552
12	30	0	1.046199049	1.130282412	1.390294934	1.484019341
13	21	0	0.964559561	1.013956706	0.908154926	0.859605334
14	21	0	0.994389242	1.188199677	1.463003731	1.220584708
15	21	0	1.131459931	1.210886383	1.332329992	1.25290354
16	21	0	1.218693063	1.186518291	1.392106317	1.334965947
17	21	0	0.987113526	1.137139325	1.077938554	0.927912755
18	30	0	1.070138391	1.049846854	0.742996996	0.703568803
19	30	0	0.832906557	0.849878197	0.922444992	1.048389104
20	30	0	1.050496839	1.125261235	1.314537553	1.358809592
21	7	2	0.544718986	0.631417381	0.54765781	0.539603827
23	7	2	1.183662816	1.183664531	1.472925006	1.472923977
24	7	2	1.307211759	1.248501821	1.196299965	1.186160885
25	30	2	1.09349086	1.09349086	1.673288113	1.791888595
26	30	3	0.824994919	0.833842261	0.647010161	0.621010761
27	21	3	1.443398194	1.415144598	1.195271994	1.22352559
28	30	3	1.319928484	1.319928484	1.334353006	1.278301661
29	30	2	0.911984521	0.911984521	0.97868876	0.988867971
30	7	3	1.10327322	1.115400671	1.142619093	0.930627257
31	30	2	1.402391515	1.402391515	1.247345225	1.247345225
32	21	3	0.886297251	0.886297251	0.709521225	0.709521225
33	21	2	1.191558676	1.253194747	1.249833004	1.426863879
34	21	3	1.527466122	1.533202797	1.145199139	1.210335182
35	21	2	1.307528348	1.307528348	1.558471607	1.558471607
71	0	0	1.471080352	1.604421259	0.797530566	1.016465408
72	0	0	1.556690408	1.520386602	1.201715935	1.238032089
73	0	0	1.343344065	0.970728759	1.095880541	1.082506971
74	0	0	1.473647021	1.54907375	1.944772613	1.668197993
75	0	0	1.524192873	1.473609634	1.521569609	1.573766663
76	7	0	1.359934632	1.441763456	1.939844046	1.735557362
77	7	0	1.136953762	1.136953762	1.098819022	1.098819022
78	7	0	1.022548856	0.972636868	1.119703263	1.169615251
79	7	0	1.138181359	1.138181359	1.217453118	1.217453118
80	7	0	1.519846377		0.950601862	
81	21	0	1.18979497	1.197695289	1.11117594	1.014591942
82	21	0	1.282692404	1.148876099	0.47338459	0.597360225
83	21	0	1.084186985	1.084186985	0.937520871	0.937520871
84	30	0	0.929754665	0.928909856	1.129624195	1.130469004
85	30	0	0.873846008	0.873846008	0.919537038	1.032461556
86	30	0	0.896889091	0.910439649	1.068177803	0.986529054
87	21	0	1.062296725	1.000413008	0.733519906	0.774343766
88	21	0	1.179429853	1.038862622	0.944472452	1.090313994
89	30	0	1.029693546	1.029693546	0.75821522	1.138074
90	30	0	0.963516155	1.054755527	1.123742431	1.032503059

Sample	Day	Class	Total ant (mm3)	ReTotalant (mm3)	Total post (mm3)	Re Total post (mm3)
91	7	3	1.135600284	1.111645164	1.020055932	1.035419245
92	7	3	1.286396461	1.502780412	1.600305259	1.500896999
93	30	3	1.010253335	1.006476562	0.540246952	0.635673668
94	30	2	1.075348904	1.102090213	1.862923552	1.836182243
95	7	3	1.304717806	1.211912982	1.265246052	1.155367031
96	30	3	1.1987507	1.187193658	1.031806083	1.034352515
97	30	3	0.92711528	0.892826256	0.673930516	0.709751035
98	7	3	0.88028549	1.132963986	1.181332131	1.053338251
99	30	2	1.285826738	1.255419102	1.32248109	1.261130052
100	21	3	0.938828387	0.938828387	0.804562409	0.716897097
101	21	3	1.356979687	1.353992157	1.032485566	0.956880134
102	21	3	0.820817179	0.896882231	0.597816415	0.611636228
103	21	2	1.358014175	1.385186635	1.179808868	1.400720076
104	7	3	1.195891452	1.228768002	1.207916346	0.874109089
105	21	2	1.219014797	1.238359997	1.339598505	1.401374177
1	0	0	1.533463875	1.647360875	1.855393625	1.887700125
22	7	3	0.51033225	0.555335625	1.540428875	1.381756125

Note: GLD004, 009 and 010 Not Included in study due to issues with imaging process

Sample	Day	Class	Cart ant (mm3)	Re Cart ant (mm3	Cart post (mm3)	Re Cart post mm3)
2	0	0	0.782957868	0.73835209	0.727104434	0.753813501
3	0	0	0.713947297	0.550443999	0.917091791	0.728125545
5	0	0	0.587965455	0.587965455	0.693612885	0.693393708
6	7	0	0.462228172	0.462228172	0.198944459	0.198944459
7	7	0	0.446348301	0.484492988	0.635610556	0.597465869
8	7	0	0.40716158	0.44432563	0.277420801	0.284068141
11	30	0	0.551109762	0.551109762	0.447964517	0.447964517
12	30	0	0.393323931	0.357734251	0.576208444	0.660663276
13	21	0	0.420977963	0.509039097	0.276368134	0.31232551
14	21	0	0.497553056	0.497615825	0.180131595	0.29390298
15	21	0	0.30884406	0.30884406	0.346397758	0.346397758
16	21	0	0.399590198	0.328423872	0.208071003	0.279237329
17	21	0	0.40512759	0.40512759	0.404150383	0.404150383
18	30	0	0.552715688	0.552619648	0.191046198	0.191142238
19	30	0	0.357572698	0.357572698	0.412191675	0.440575268
20	30	0	0.369305356	0.369258365	0.138856004	0.20204072
21	7	2	0.197803984	0.236794852	0.27901678	0.240403555
23	7	2	0.45357634	0.479257436	0.539709814	0.570130827
24	7	2	0.402481688	0.578746644	0.428008434	0.3593954
25	30	2	0.295929767	0.39967046	0.763382858	0.791587405
26	30	3	0.483665329	0.422823646	0.196867251	0.343568694
27	21	3	0.439321946	0.439321946	0.654650829	0.723564331
28	30	3	0.535445981	0.520024358	0.491044974	0.506466597
29	30	2	0.522002782	0.418638703	0.154313985	0.308965482
30	7	3	0.631193059	0.51912021	0.180611452	0.291986639
31	30	2	0.500976882	0.55227802	0.535662757	0.512779512
32	21	3	0.291558232	0.291558232	0.49029792	0.449135862
33	21	2	0.310790242	0.367327961	0.519853887	0.739753931
34	21	3	0.636639556	0.636647102	0.530454988	0.495731726
35	21	2	0.435402485	0.435402485	0.576355248	0.671679407
71	0	0	0.43979117	0.478586871	0.476672588	0.21999334
72	0	0	0.638139838	0.621601064	0.59148292	0.638053402
73	0	0	0.491924769	0.492212203	0.523425546	0.523138112
74	0	0	0.474720232	0.478138913	0.780421383	0.751395008
75	0	0	0.548425444	0.503907817	0.543221105	0.587739418
76	7	0	0.568122219	0.568122219	0.714933079	0.714933079
77	7	0	0.499049565	0.499049565	0.538743583	0.538743583
78	7	0	0.483073311	0.483073311	0.530488602	0.530488602
79	7	0	0.240493421	0.240493421	0.521769199	0.521769199
80	7	0	0.430661539		0.322276626	
81	21	0	0.375822699	0.375822699	0.496477751	0.374539193
82	21	0	0.351680987	0.351680987	0.495850747	0.495850747
83	21	0	0.587835115	0.738318133	0.183409989	0.032926971
84	30	0	0.356336526	0.427597177	0.599265933	0.528005282
85	30	0	0.318604811	0.354840703	0.41761279	0.381376898
86	30	0	0.244880048	0.244880048	0.667370641	0.667370641
87	21	0	0.391825364	0.391825364	0.312166015	0.22425683
88	21	0	0.583314375	0.541018359	0.259054866	0.383458565
89	30	0	0.471784838	0.444825724	0.368898558	0.395816169
90	30	0	0.494122713	0.494122713	0.410100061	0.410100061

Sample	Day	Class	Cart ant (mm3)	Re Cart ant (mm3	Cart post (mm3)	Re Cart post mm3)
91	7	3	0.559296829	0.559296829	0.448124012	0.390696894
92	7	3	0.465105256	0.343908607	0.563828202	0.595324863
93	30	3	0.367511123	0.367511123	0.334949104	0.334949104
94	30	2	0.390469485	0.331665222	0.818523881	0.877328144
95	7	3	0.479074274	0.526361283	0.589413944	0.569969617
96	30	3	0.366324343	0.366324343	0.630136276	0.619885035
97	30	3	0.433344485	0.433385988	0.476270249	0.509202708
98	7	3	0.483335706	0.483335706	0.439879321	0.647305484
99	30	2	0.552563053	0.468232387	0.576825158	0.584486749
100	21	3	0.318384605	0.318384605	0.394492532	0.394492532
101	21	3	0.467396496	0.46725175	0.477286901	0.475317738
102	21	3	0.376685344	0.376685344	0.299170088	0.299170088
103	21	2	0.428198456	0.493888787	0.401077789	0.415254322
104	7	3	0.487170789	0.417164489	0.53572827	0.621153106
105	21	2	0.493925145	0.425893839	0.309577737	0.522690154
1	0	0	0.511465625	0.525523125	0.54300875	0.59131225
22	7	3	0.28549875	0.251228625	0.524039875	0.842963875

Note: GLD004, 009 and 010 Not Included in study due to issues with imaging process

Sample	Day	Class	ML (um)	SI (um)	AP (um)	½ AP (um) -ant
2	0	0	1561	1911	2891	1449
3	0	0	1694	1911	2730	1372
5	0	0	1337	1792	2891	1449
6	7	0	1253	1673	2415	1211
7	7	0	1428	1820	2555	1281
8	7	0	1421	1708	2807	1407
11	30	0	1330	1841	2954	1484
12	30	0	1379	1799	2870	1442
13	21	0	1274	1624	2492	1253
14	21	0	1379	1750	2674	1344
15	21	0	1393	1820	2534	1274
16	21	0	1330	1659	2968	1491
17	21	0	1386	1750	2226	1120
18	30	0	1218	1701	2492	1253
19	30	0	1295	1624	2562	1288
20	30	0	1372	1715	2786	1400
21	7	2	917	1708	1869	938
23	7	2	1330	1792	2520	1267
24	7	2	1232	1680	2681	1344
25	30	2	1316	1757	3416	1715
26	30	3	1197	1603	2086	1050
27	21	3	1393	1911	2562	1288
28	30	3	1281	1813	2786	1400
29	30	2	1267	1617	2485	1246
30	7	3	1414	1701	2730	1372
31	30	2	1449	1869	2975	1491
32	21	3	1099	1659	2156	1085
33	21	2	1386	1687	2926	1470
34	21	3	1477	1757	3017	1512
35	21	2	1442	1673	3052	1533
71	0	0	1484	1792	2492	1253
72	0	0	1456	1820	2730	1372
73	0	0	1316	1750	2639	1323
74	0	0	1414	1806	2660	1337
75	0	0	1470	1792	2513	1260
76	7	0	1386	1890	2968	1491
77	7	0	1169	1645	2632	1323
78	7	0	1204	1645	2674	1344
79	7	0	1176	1799	2968	1491
80	7	0	1463	1799	2674	1344
81	21	0	1358	1799	2786	1400
82	21	0	1218	1771	2576	1295
83	21	0	1267	1757	2730	1372
84	30	0	1302	1624	2730	1372
85	30	0	1155	1582	2646	1330
86	30	0	1169	1659	2639	1323
87	21	0	1288	1645	1988	1001
88	21	0	1162	1722	2828	1421
89	30	0	1239	1673	2534	1274

Sample	Day	Class	ML (um)	SI (um)	AP (um)	½ AP (um) -ant
90	30	0	1169	1645	3108	1561
91	7	3	1351	1694	2660	1337
92	7	3	1323	1841	2786	1400
93	30	3	1085	1764	1974	994
94	30	2	1400	1820	3213	1610
95	7	3	1323	1631	2576	1295
96	30	3	1379	1687	2807	1407
97	30	3	1162	1589	2121	1064
98	7	3	1281	1722	2618	1316
99	30	2	1379	1757	2569	1288
100	21	3	917	1603	2177	1092
101	21	3	1379	1757	2429	1218
102	21	3	1169	1575	2142	1078
103	21	2	1281	1596	2667	1337
104	7	3	1253	1701	2464	1239
105	21	2	1358	1722	2569	1288
1	0	0	1395	1805	2810	1410
22	7	3	1275	1815	2340	1175

Note: GLD004, 009 and 010 Not Included in study due to issues with imaging process

Sample	Day	Class	Total cube (mm3)	Ant Cube (mm3)	Post Cube (mm3)
2	0	0	8.62405826	4.32246988	4.30158838
3	0	0	8.83764882	4.44148505	4.39616377
5	0	0	6.92655846	3.4716649	3.45489357
6	7	0	5.06248964	2.53858176	2.52390788
7	7	0	6.6403428	3.32926776	3.31107504
8	7	0	6.81277988	3.41488468	3.3978952
11	30	0	7.23295762	3.63361852	3.5993391
12	30	0	7.11995627	3.57734388	3.54261239
13	21	0	5.15588819	2.59242693	2.56346126
14	21	0	6.4530305	3.243408	3.2096225
15	21	0	6.42434884	3.22992124	3.1944276
16	21	0	6.54880296	3.28984677	3.25895619
17	21	0	5.399163	2.71656	2.682603
18	30	0	5.16297046	2.59598795	2.5669825
19	30	0	5.38809096	2.70876704	2.67932392
20	30	0	6.55540228	3.294172	3.26123028
21	7	2	2.92729508	1.46912937	1.45816572
23	7	2	6.0060672	3.01971712	2.98635008
24	7	2	5.54902656	2.78175744	2.76726912
25	30	2	7.89851619	3.96544358	3.93307261
26	30	3	4.00259803	2.01473055	1.98786748
27	21	3	6.82010293	3.42868562	3.3914173
28	30	3	6.47035406	3.2514342	3.21891986
29	30	2	5.09111642	2.55272879	2.53838762
30	7	3	6.56623422	3.29995361	3.26628061
31	30	2	8.05683848	4.03789787	4.0189406
32	21	3	3.9309076	1.97821649	1.95269111
33	21	2	6.84152053	3.43712754	3.40439299
34	21	3	7.82938351	3.92377457	3.90560895
35	21	2	7.36284623	3.69831038	3.66453585
71	0	0	6.62704538	3.33213798	3.29490739
72	0	0	7.2342816	3.63569024	3.59859136
73	0	0	6.077617	3.046869	3.030748
74	0	0	6.79279944	3.41427551	3.37852393
75	0	0	6.61984512	3.3191424	3.30070272
76	7	0	7.77479472	3.90573414	3.86906058
77	7	0	5.06134916	2.54413562	2.51721355
78	7	0	5.29607092	2.66189952	2.6341714
79	7	0	6.27917203	3.15439538	3.12477665
80	7	0	7.03779954	3.53732333	3.50047621
81	21	0	6.80631501	3.4202588	3.38605621
82	21	0	5.55663293	2.79341601	2.76321692
83	21	0	6.07730487	3.05423527	3.0230696
84	30	0	5.77244304	2.90102266	2.87142038
85	30	0	4.83479766	2.4301893	2.40460836
86	30	0	5.11800007	2.56578783	2.55221224
87	21	0	4.21209488	2.12087876	2.09121612
88	21	0	5.65872619	2.84336984	2.81535635
89	30	0	5.2525943	2.64080708	2.61178722

Sample	Day	Class	Total cube (mm3)	Ant Cube (mm3)	Post Cube (mm3)
90	30	0	5.97669954	3.00181081	2.97488874
91	7	3	6.08766004	3.05985018	3.02780986
92	7	3	6.7857014	3.4099002	3.3758012
93	30	3	3.77811756	1.90245636	1.8756612
94	30	2	8.186724	4.10228	4.084444
95	7	3	5.55852629	2.79436784	2.76415845
96	30	3	6.53012901	3.27320681	3.2569222
97	30	3	3.91625258	1.96458875	1.95166383
98	7	3	5.77499908	2.90294071	2.87205836
99	30	2	6.22443781	3.12069906	3.10373874
100	21	3	3.20008333	1.60518649	1.59489684
101	21	3	5.88523139	2.95109585	2.93413553
102	21	3	3.94379685	1.98478665	1.9590102
103	21	2	5.45261749	2.73346441	2.71915308
104	7	3	5.25165379	2.64074637	2.61090743
105	21	2	6.00754484	3.01195709	2.99558776
1	0	0	7.07550975	3.55034475	3.525165
22	7	3	5.4150525	2.71909688	2.69595563

Note: GLD004, 009 and 010 Not Included in study due to issues with imaging process

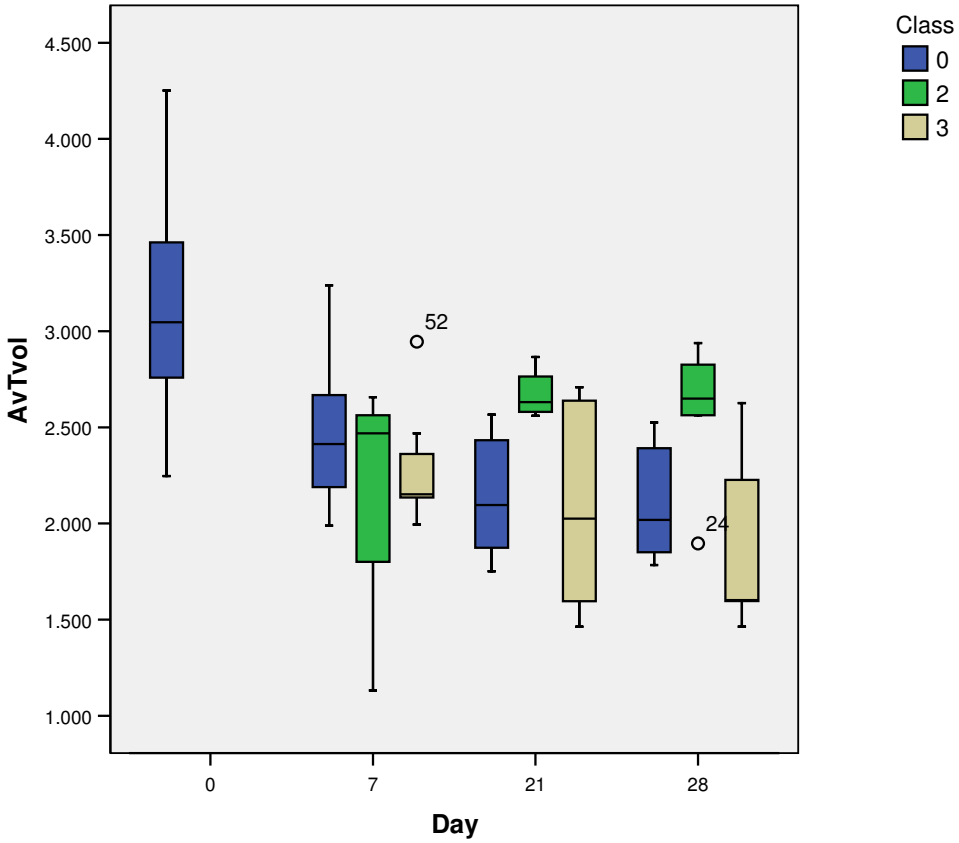
Sample	Day	Class	**Weight @ Day 0**	**Weight @ removal**	**Weight @ sacrifice**
2	0	0		NA	49.1
3	0	0		NA	48.4
4	0	0		NA	43.9
5	7	0		NA	45.7
7	7	0		NA	54.2
8	7	0		NA	45.1
11	30	0		NA	45.9
12	30	0		NA	43.2
13	21	0		NA	44.8
14	21	0		NA	45.6
15	21	0		NA	47.7
16	21	0		NA	47.6
17	21	0		NA	45.9
18	30	0		NA	38.6
19	30	0		NA	42.3
20	30	0		NA	48.8
21	7	2		NA	33.4
23	7	2		NA	41.1
24	7	2		NA	41.1
25	30	2	44.6	39.3	39.6
26	30	3	46.2	39.2	37.3
27	21	3		NA	35.9
28	30	3	45.2	37.8	36.9
29	30	2	44.5	37.2	35.7
30	7	3		NA	36.1
31	30	2	43.2	37.8	37.0
32	21	3		NA	31.5
33	21	2		NA	36.5
34	21	3		NA	43.2
35	21	2		NA	38.3
71	0	0		NA	44.8
72	0	0		NA	46.2
73	0	0		NA	42.2
74	0	0		NA	42.8
75	0	0		NA	41.8
76	7	0		NA	45.9
77	7	0		NA	44.2
78	7	0		NA	40.9
79	7	0		NA	42.2
80	7	0		NA	41.5
81	21	0		NA	40.3
82	21	0		NA	41.4
83	21	0		NA	37.8
84	30	0		NA	42.7
85	30	0		NA	40.0
86	30	0		NA	49.5
87	21	0		NA	36.1
88	21	0		NA	37.8
89	30	0		NA	38.9
90	30	0		NA	37.6

Sample	Day	Class	**Weight @ Day 0**	**Weight @ removal**	**Weight @ sacrifice**
91	7	3		NA	39.2
92	7	3		NA	39.8
93	30	3	40.6	35.6	38.4
94	30	2	38.3	34.7	40.3
95	7	3		NA	27.7
96	30	3	36.1	32.0	37.2
97	30	3	32.9	26.9	31.8
98	7	3		NA	36.3
99	30	2	38.8	36.1	41.9
100	21	3		NA	33.4
101	21	3		NA	34.4
102	21	3		NA	26.1
103	21	2		NA	34.4
104	7	3		NA	39.2
105	21	2		NA	31.4
1	0	0		NA	51.7
22	7	3		NA	33.3

Note: GLD004, 009 and 010 Not Included in study due to issues with imaging process

15.7Statistical Analyses

15.7.1 Univariate Analysis of Variance - AvTvol



Between-Subjects Factors

		N
Day	0	9
	7	18
	21	20
	28	20
Class	0	37
	2	12
	3	18

Tests of Between-Subjects Effects

Dependent Variable: AvTvol

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	16.448[a]	6	2.741	51.098	.000
Intercept	.425	1	.425	7.928	.007
Day	1.852	3	.617	11.508	.000
Class	.232	2	.116	2.165	.124
Tcube	8.923	1	8.923	166.318	.000
Error	3.219	60	.054		
Total	391.866	67			
Corrected Total	19.667	66			

a. R Squared = .836 (Adjusted R Squared = .820)

Estimated Marginal Means

Day

Estimates

Dependent Variable: AvTvol

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	2.813[a]	.092	2.629	2.996
7	2.417[a]	.056	2.304	2.529
21	2.335[a]	.053	2.228	2.442
28	2.218[a]	.053	2.112	2.324

a. Covariates appearing in the model are evaluated at the following values: Tcube = 6.04235.

Pairwise Comparisons

Dependent Variable: AvTvol

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.396*	.105	.002	.110	.682
	21	.477*	.104	.000	.195	.760
	28	.594*	.103	.000	.314	.875
7	0	-.396*	.105	.002	-.682	-.110
	21	.081	.076	1.000	-.125	.287
	28	.198	.076	.068	-.009	.405
21	0	-.477*	.104	.000	-.760	-.195
	7	-.081	.076	1.000	-.287	.125
	28	.117	.073	.695	-.083	.318
28	0	-.594*	.103	.000	-.875	-.314
	7	-.198	.076	.068	-.405	.009
	21	-.117	.073	.695	-.318	.083

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable: AvTvol

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	1.852	3	.617	11.508	.000
Error	3.219	60	.054		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Class

Estimates

Dependent Variable: AvTvol

Class	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	2.372[a]	.039	2.294	2.449
2	2.539[a]	.072	2.394	2.683
3	2.427[a]	.060	2.307	2.546

a. Covariates appearing in the model are evaluated at the following values: Tcube = 6.04235.

Pairwise Comparisons

Dependent Variable: AvTvol

(I) Class	(J) Class	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	2	-.167	.081	.127	-.365	.031
	3	-.055	.072	1.000	-.232	.121
2	0	.167	.081	.127	-.031	.365
	3	.112	.090	.652	-.109	.333
3	0	.055	.072	1.000	-.121	.232
	2	-.112	.090	.652	-.333	.109

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

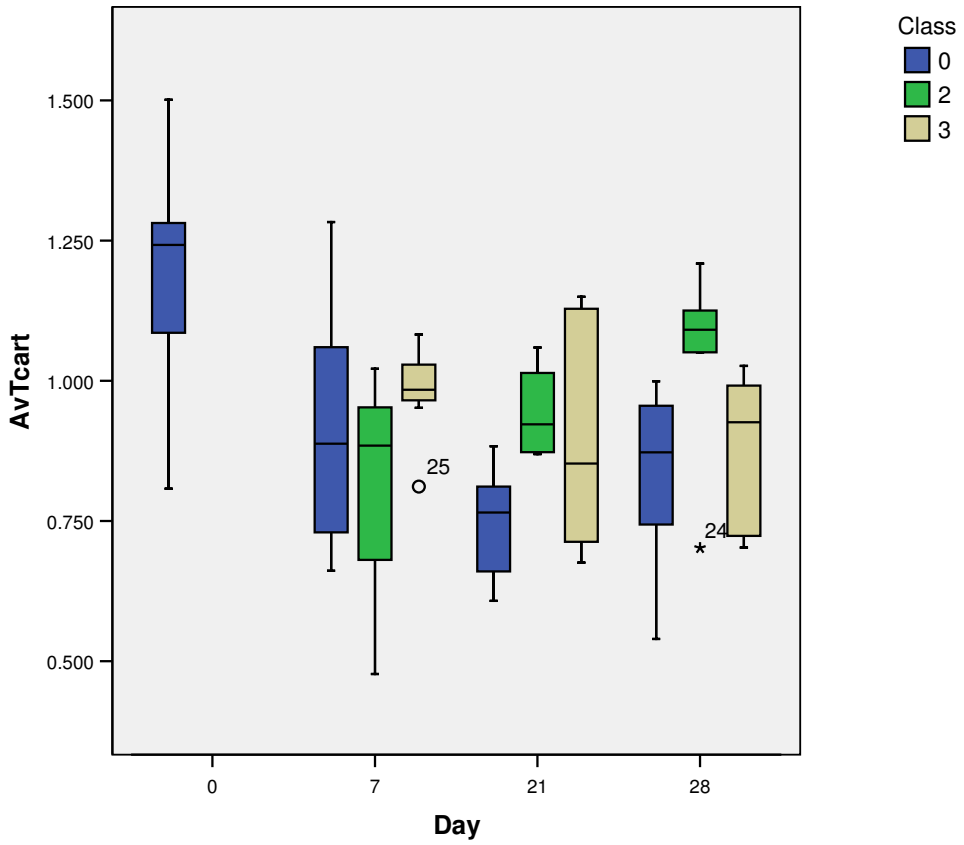
Univariate Tests

Dependent Variable: AvTvol

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.232	2	.116	2.165	.124
Error	3.219	60	.054		

The F tests the effect of Class. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.7.2 Univariate Analysis of Variance - AvTcart



Between-Subjects Factors

		N
Day	0	9
	7	18
	21	20
	28	20
Class	0	37
	2	12
	3	18

Tests of Between-Subjects Effects

Dependent Variable: AvTcart

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.753[a]	6	.292	16.211	.000
Intercept	.299	1	.299	16.571	.000
Day	.386	3	.129	7.142	.000
Class	.216	2	.108	5.995	.004
Tcube	.788	1	.788	43.713	.000
Error	1.081	60	.018		
Total	60.050	67			
Corrected Total	2.834	66			

a. R Squared = .618 (Adjusted R Squared = .580)

Estimated Marginal Means

Day

Estimates

Dependent Variable: AvTcart

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.152[a]	.053	1.045	1.258
7	.940[a]	.032	.875	1.005
21	.875[a]	.031	.813	.937
28	.927[a]	.031	.866	.989

a. Covariates appearing in the model are evaluated at the following values: Tcube = 6.04235.

Pairwise Comparisons

Dependent Variable: AvTcart

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.211*	.061	.001	.090	.333
	21	.276*	.060	.000	.156	.396
	28	.224*	.060	.000	.105	.343
7	0	-.211*	.061	.001	-.333	-.090
	21	.065	.044	.143	-.023	.153
	28	.013	.044	.769	-.075	.101
21	0	-.276*	.060	.000	-.396	-.156
	7	-.065	.044	.143	-.153	.023
	28	-.052	.043	.226	-.137	.033
28	0	-.224*	.060	.000	-.343	-.105
	7	-.013	.044	.769	-.101	.075
	21	.052	.043	.226	-.033	.137

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Univariate Tests

Dependent Variable: AvTcart

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.386	3	.129	7.142	.000
Error	1.081	60	.018		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Class

Estimates

Dependent Variable: AvTcart

Class	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	.900[a]	.022	.856	.945
2	.980[a]	.042	.896	1.063
3	1.041[a]	.035	.972	1.110

a. Covariates appearing in the model are evaluated at the following values: Tcube = 6.04235.

Pairwise Comparisons

Dependent Variable: AvTcart

(I) Class	(J) Class	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	2	-.079	.047	.094	-.173	.014
	3	-.141*	.042	.001	-.224	-.058
2	0	.079	.047	.094	-.014	.173
	3	-.061	.052	.243	-.166	.043
3	0	.141*	.042	.001	.058	.224
	2	.061	.052	.243	-.043	.166

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

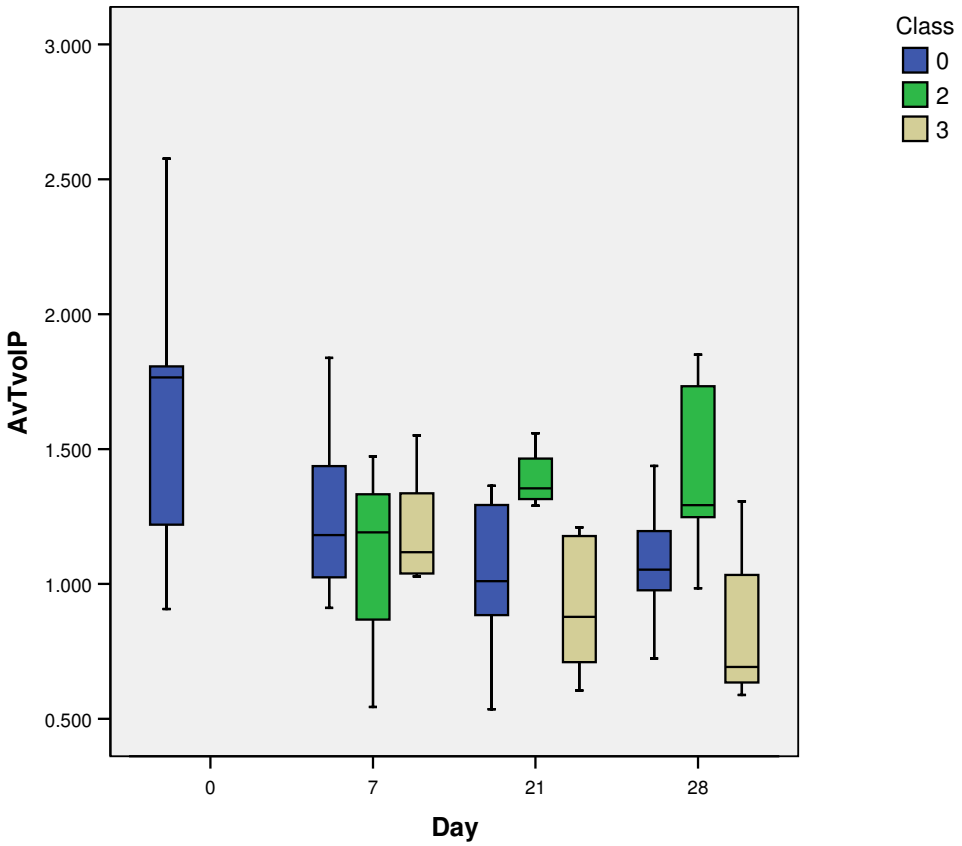
Univariate Tests

Dependent Variable: AvTcart

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.216	2	.108	5.995	.004
Error	1.081	60	.018		

The F tests the effect of Class. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.7.3 Univariate Analysis of Variance - AvTvolP



Between-Subjects Factors

		N
Day	0	9
	7	18
	21	20
	28	20
Class	0	37
	2	12
	3	18

Tests of Between-Subjects Effects

Dependent Variable: AvTvolP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6.447[a]	6	1.074	22.511	.000
Intercept	.000	1	.000	.006	.939
Day	.553	3	.184	3.861	.014
Class	.197	2	.098	2.061	.136
Tcube	3.542	1	3.542	74.220	.000
Error	2.864	60	.048		
Total	104.001	67			
Corrected Total	9.310	66			

a. R Squared = .692 (Adjusted R Squared = .662)

Estimated Marginal Means

Day

Estimates

Dependent Variable: AvTvolP

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.424[a]	.087	1.251	1.597
7	1.262[a]	.053	1.157	1.368
21	1.134[a]	.050	1.033	1.235
28	1.145[a]	.050	1.045	1.245

a. Covariates appearing in the model are evaluated at the following values: Tcube = 6.04235.

Pairwise Comparisons

Dependent Variable: AvTvolP

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.162	.099	.642	-.108	.432
	21	.290*	.098	.026	.023	.557
	28	.279*	.097	.033	.015	.544
7	0	-.162	.099	.642	-.432	.108
	21	.128	.071	.465	-.066	.323
	28	.117	.072	.641	-.078	.313
21	0	-.290*	.098	.026	-.557	-.023
	7	-.128	.071	.465	-.323	.066
	28	-.011	.069	1.000	-.200	.178
28	0	-.279*	.097	.033	-.544	-.015
	7	-.117	.072	.641	-.313	.078
	21	.011	.069	1.000	-.178	.200

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable: AvTvolP

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.553	3	.184	3.861	.014
Error	2.864	60	.048		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Class

Estimates

Dependent Variable: AvTvolP

Class	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.195[a]	.036	1.122	1.268
2	1.340[a]	.068	1.204	1.476
3	1.190[a]	.056	1.077	1.302

a. Covariates appearing in the model are evaluated at the following values: Tcube = 6.04235.

Pairwise Comparisons

Dependent Variable: AvTvolP

(I) Class	(J) Class	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	2	-.145	.076	.183	-.332	.042
	3	.005	.068	1.000	-.162	.171
2	0	.145	.076	.183	-.042	.332
	3	.150	.085	.246	-.059	.358
3	0	-.005	.068	1.000	-.171	.162
	2	-.150	.085	.246	-.358	.059

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

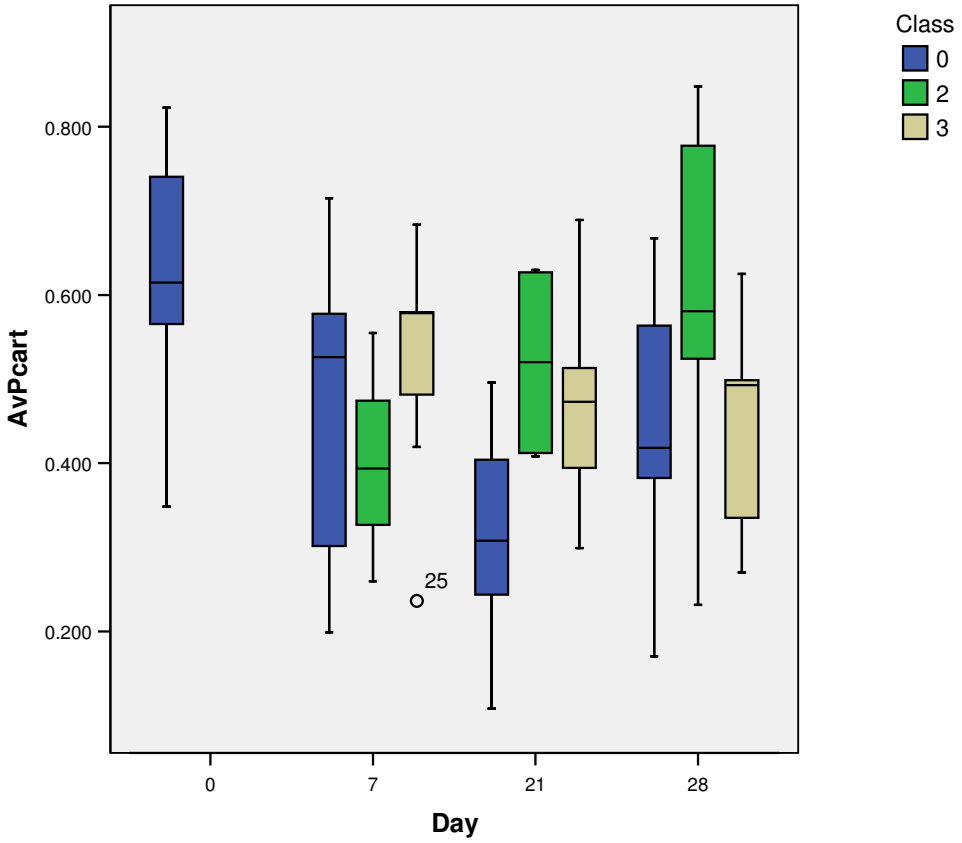
Univariate Tests

Dependent Variable: AvTvolP

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.197	2	.098	2.061	.136
Error	2.864	60	.048		

The F tests the effect of Class. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.7.4 Univariate Analysis of Variance – AvPcart



Between-Subjects Factors

		N
Day	0	9
	7	18
	21	20
	28	20
Class	0	37
	2	12
	3	18

Tests of Between-Subjects Effects

Dependent Variable: AvPcart

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.805[a]	6	.134	6.980	.000
Intercept	.030	1	.030	1.566	.216
Day	.174	3	.058	3.018	.037
Class	.173	2	.087	4.504	.015
Tcube	.347	1	.347	18.060	.000
Error	1.153	60	.019		
Total	16.984	67			
Corrected Total	1.957	66			

a. R Squared = .411 (Adjusted R Squared = .352)

Estimated Marginal Means

Day

Estimates

Dependent Variable: AvPcart

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	.623[a]	.055	.513	.733
7	.495[a]	.034	.428	.563
21	.438[a]	.032	.374	.502
28	.498[a]	.032	.435	.562

a. Covariates appearing in the model are evaluated at the following values: Tcube = 6.04235.

Pairwise Comparisons

Dependent Variable: AvPcart

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.128	.063	.277	-.044	.299
	21	.185*	.062	.024	.016	.355
	28	.125	.062	.281	-.043	.293
7	0	-.128	.063	.277	-.299	.044
	21	.058	.045	1.000	-.066	.181
	28	-.003	.045	1.000	-.127	.121
21	0	-.185*	.062	.024	-.355	-.016
	7	-.058	.045	1.000	-.181	.066
	28	-.061	.044	1.000	-.180	.059
28	0	-.125	.062	.281	-.293	.043
	7	.003	.045	1.000	-.121	.127
	21	.061	.044	1.000	-.059	.180

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable: AvPcart

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.174	3	.058	3.018	.037
Error	1.153	60	.019		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Class

Estimates

Dependent Variable: AvPcart

Class	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	.442[a]	.023	.396	.488
2	.540[a]	.043	.454	.627
3	.559[a]	.036	.487	.630

a. Covariates appearing in the model are evaluated at the following values: Tcube = 6.04235.

Pairwise Comparisons

Dependent Variable: AvPcart

(I) Class	(J) Class	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	2	-.098	.048	.138	-.217	.021
	3	-.117*	.043	.025	-.222	-.011
2	0	.098	.048	.138	-.021	.217
	3	-.019	.054	1.000	-.151	.114
3	0	.117*	.043	.025	.011	.222
	2	.019	.054	1.000	-.114	.151

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable: AvPcart

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.173	2	.087	4.504	.015
Error	1.153	60	.019		

The F tests the effect of Class. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.7.5 Univariate Analysis of Variance - ML

Between-Subjects Factors

		N
Day	0	9
	7	18
	21	20
	28	20
Class	0	37
	2	12
	3	18

Tests of Between-Subjects Effects

Dependent Variable: ML

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	876345.148[a]	6	146057.525	32.783	.000
Intercept	1274250.656	1	1274250.656	286.011	.000
Day	30987.282	3	10329.094	2.318	.084
Class	4875.881	2	2437.941	.547	.581
Tcube	625513.162	1	625513.162	140.399	.000
Error	267314.703	60	4455.245		
Total	116266505	67			
Corrected Total	1143659.851	66			

a. R Squared = .766 (Adjusted R Squared = .743)

Estimated Marginal Means

Day

Estimates

Dependent Variable: ML

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1360.450[a]	26.457	1307.529	1413.371
7	1300.431[a]	16.153	1268.120	1332.742
21	1320.279[a]	15.397	1289.481	1351.077
28	1287.811[a]	15.244	1257.317	1318.304

a. Covariates appearing in the model are evaluated at the following values: Tcube = 6.04235.

Pairwise Comparisons

Dependent Variable: ML

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	60.019	30.232	.310	-22.470	142.508
	21	40.171	29.852	1.000	-41.281	121.623
	28	72.639	29.629	.103	-8.204	153.483
7	0	-60.019	30.232	.310	-142.508	22.470
	21	-19.848	21.786	1.000	-79.292	39.596
	28	12.620	21.873	1.000	-47.061	72.302
21	0	-40.171	29.852	1.000	-121.623	41.281
	7	19.848	21.786	1.000	-39.596	79.292
	28	32.468	21.159	.781	-25.265	90.202
28	0	-72.639	29.629	.103	-153.483	8.204
	7	-12.620	21.873	1.000	-72.302	47.061
	21	-32.468	21.159	.781	-90.202	25.265

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable: ML

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	30987.282	3	10329.094	2.318	.084
Error	267314.7	60	4455.245		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

Class

Estimates

Dependent Variable: ML

Class	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1314.308[a]	11.121	1292.063	1336.554
2	1306.050[a]	20.799	1264.445	1347.655
3	1331.370[a]	17.188	1296.989	1365.750

a. Covariates appearing in the model are evaluated at the following values: Tcube = 6.04235.

Pairwise Comparisons

Dependent Variable: ML

(I) Class	(J) Class	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	2	8.258	23.198	1.000	-48.878	65.394
	3	-17.062	20.642	1.000	-67.902	33.779
2	0	-8.258	23.198	1.000	-65.394	48.878
	3	-25.320	25.879	.995	-89.058	38.418
3	0	17.062	20.642	1.000	-33.779	67.902
	2	25.320	25.879	.995	-38.418	89.058

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable: ML

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	4875.881	2	2437.941	.547	.581
Error	267314.7	60	4455.245		

The F tests the effect of Class. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.7.6 Univariate Analysis of Variance - SI

Between-Subjects Factors

		N
Day	0	9
	7	18
	21	20
	28	20
Class	0	37
	2	12
	3	18

Tests of Between-Subjects Effects

Dependent Variable: SI

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	320848.503[a]	10	32084.850	9.748	.000
Intercept	3639188.493	1	3639188.493	1105.709	.000
Day	23134.932	3	7711.644	2.343	.083
Class	6805.215	2	3402.608	1.034	.362
Tcube	196487.024	1	196487.024	59.699	.000
Day * Class	37037.333	4	9259.333	2.813	.034
Error	184311.169	56	3291.271		
Total	201406777	67			
Corrected Total	505159.672	66			

a. R Squared = .635 (Adjusted R Squared = .570)

Class * Day

Dependent Variable: SI

Class	Day	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	0	1757.986[a]	20.734	1716.452	1799.521
	7	1736.523[a]	20.332	1695.793	1777.252
	21	1741.080[a]	18.202	1704.617	1777.542
	28	1697.030[a]	18.195	1660.581	1733.479
2	0	.[a,b]	.	.	.
	7	1791.535[a]	34.170	1723.084	1859.985
	21	1649.542[a]	28.801	1591.847	1707.237
	28	1707.980[a]	26.661	1654.572	1761.389
3	0	.[a,b]	.	.	.
	7	1735.820[a]	21.700	1692.349	1779.291
	21	1751.666[a]	24.024	1703.540	1799.792
	28	1750.086[a]	26.765	1696.471	1803.702

a. Covariates appearing in the model are evaluated at the following values: Tcube = 6.04235.

b. This level combination of factors is not observed, thus the corresponding population marginal mean is not estimable.

15.7.7 Univariate Analysis of Variance - AP

Between-Subjects Factors

		N
Day	0	9
	7	18
	21	20
	28	20
Class	0	37
	2	12
	3	18

Tests of Between-Subjects Effects

Dependent Variable: AP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4355966.524[a]	6	725994.421	29.938	.000
Intercept	3724528.566	1	3724528.566	153.588	.000
Day	369802.929	3	123267.643	5.083	.003
Class	94900.870	2	47450.435	1.957	.150
Tcube	3528468.316	1	3528468.316	145.503	.000
Error	1455011.476	60	24250.191		
Total	471007053	67			
Corrected Total	5810978.000	66			

a. R Squared = .750 (Adjusted R Squared = .725)

Estimated Marginal Means

Day

Estimates

Dependent Variable: AP

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	2434.872[a]	61.724	2311.406	2558.339
7	2645.295[a]	37.686	2569.912	2720.677
21	2626.192[a]	35.922	2554.338	2698.046
28	2703.384[a]	35.566	2632.241	2774.526

a. Covariates appearing in the model are evaluated at the following values: Tcube = 6.04235.

Pairwise Comparisons

Dependent Variable: AP

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	-210.422*	70.532	.025	-402.873	-17.972
	21	-191.319*	69.645	.048	-381.350	-1.288
	28	-268.512*	69.125	.002	-457.122	-79.901
7	0	210.422*	70.532	.025	17.972	402.873
	21	19.103	50.827	1.000	-119.581	157.787
	28	-58.089	51.031	1.000	-197.328	81.150
21	0	191.319*	69.645	.048	1.288	381.350
	7	-19.103	50.827	1.000	-157.787	119.581
	28	-77.192	49.365	.739	-211.887	57.502
28	0	268.512*	69.125	.002	79.901	457.122
	7	58.089	51.031	1.000	-81.150	197.328
	21	77.192	49.365	.739	-57.502	211.887

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable: AP

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	369802.9	3	123267.643	5.083	.003
Error	1455011	60	24250.191		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.7.8 Method Error Analysis Statistics

T-Test - Tvol

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Tvol	2.35940	66	.578044	.071152
	ReTvol	2.35106	66	.535454	.065910

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Tvol & ReTvol	66	.951	.000

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	Tvol - ReTvol	.008345	.179576	.022104	-.035800	.052491	.378	65	.707

There is no significant difference between the first and second measurement taken (p-value=0.707).

T-Test – Tcart

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Tcart	.91794	66	.218401	.026883
	ReTcart	.93545	66	.206565	.025426

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Tcart & ReTcart	66	.911	.000

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	Tcart - ReTcart	-.017507	.090388	.011126	-.039727	.004714	-1.573	65	.120

There is no significant difference between the first and second measurement taken (p-value=0.120).

T-Test - TvolP

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	TvolP	1.19672	66	.399662	.049195
	ReTvolP	1.18814	66	.367737	.045265

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	TvolP & ReTvolP	66	.934	.000

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	TvolP - ReTvolP	.008585	.143247	.017632	-.026630	.043799	.487	65	.628

There is no significant difference between the first and second measurement taken (p-value=0.628).

T-Test - Pcart

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Pcart	.46614	66	.175588	.021613
	RePcart	.48562	66	.180856	.022262

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Pcart & RePcart	66	.874	.000

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	Pcart - RePcart	-.019481	.089769	.011050	-.041549	.002587	-1.763	65	.083

At the 5% significance level there is no significant difference between the first and second measurement taken (p-value=0.083).

15.7.9 Error Analysis for Multiple measurements

Errror analysis that can extend to having more than 2 measurements for the same variable on each case

Tvol Error Analysis has a Coefficient of variation of 5.37%.

Tcart Error Analysis has a Coefficient of variation of 6.83%

TvolP Error Analysis has a Coefficient of variation of 8.94%

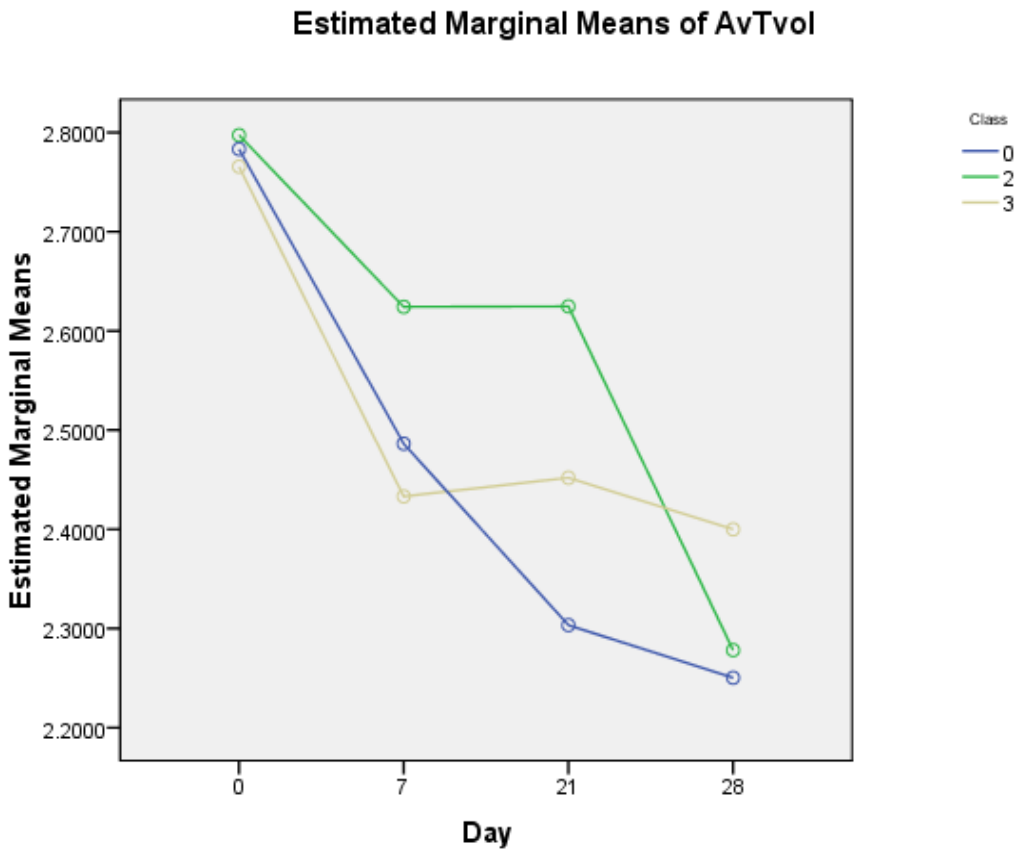
Pcart Error Analysis has a Coefficient of variation of 13.32%

Since you have at most 2 measurements for each variable of interest you can get away with the Paired Sample T test

15.8 In-depth Analysis (Class X Day interaction)

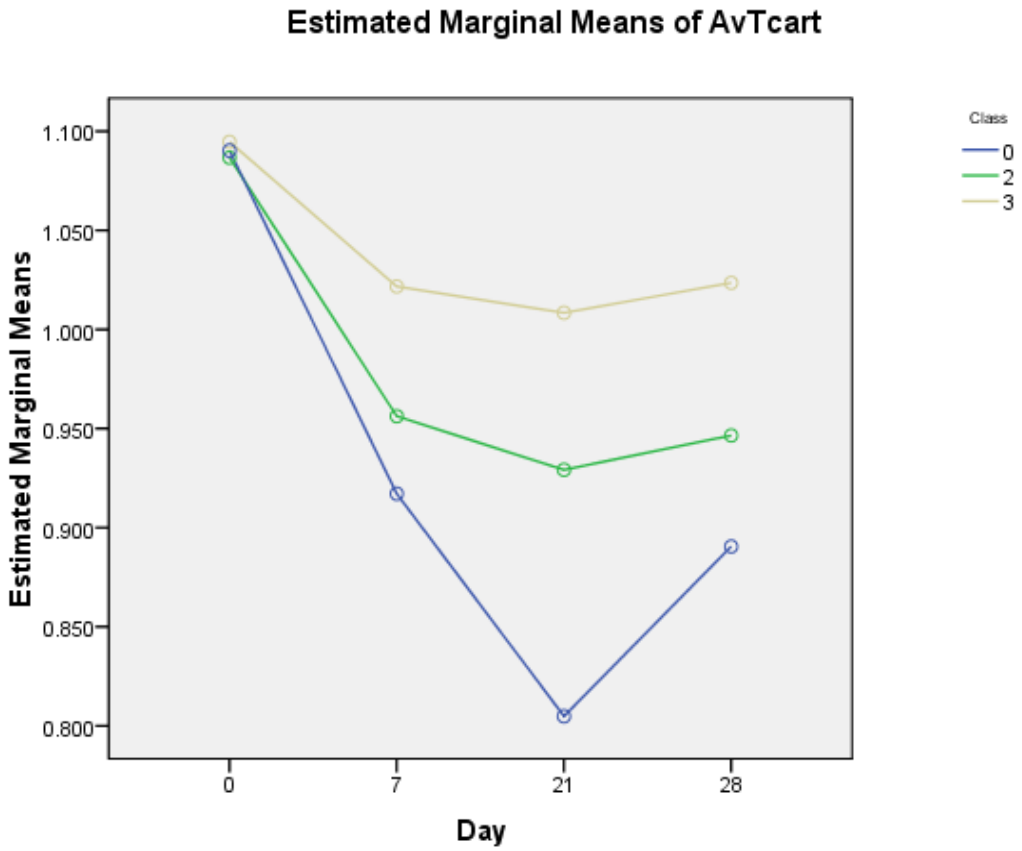
15.8.1 AvTVol

Profile Plot



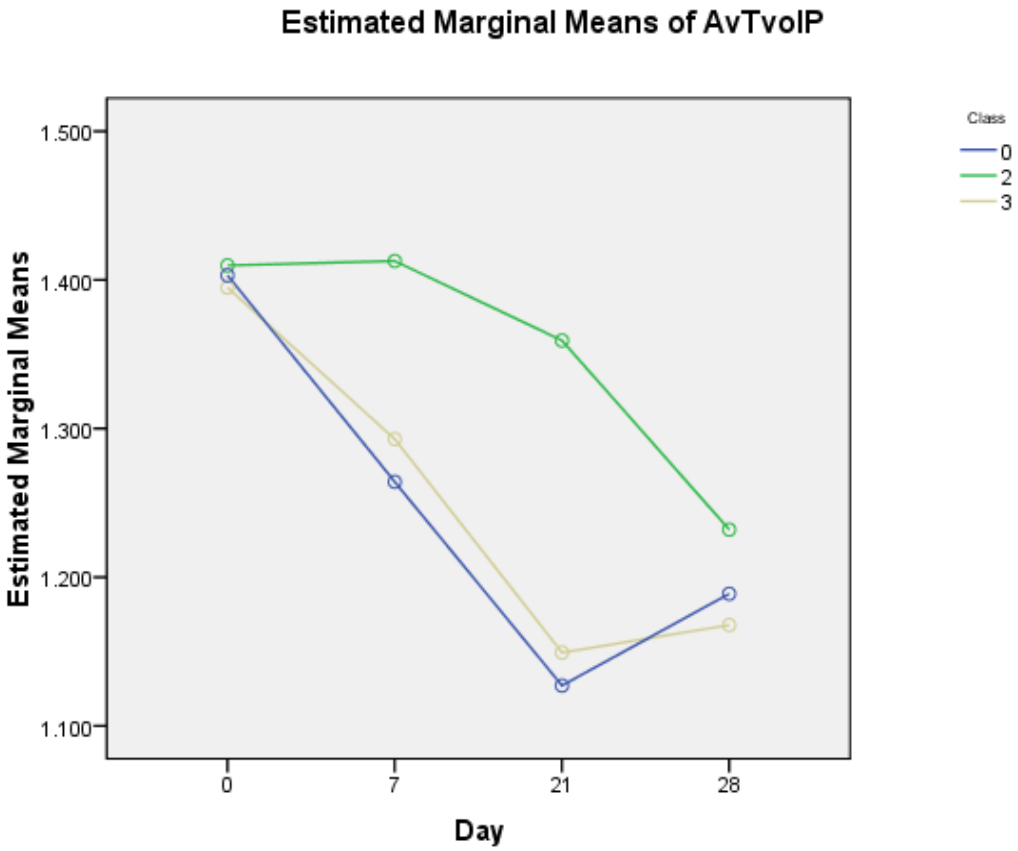
15.8.2 AvTCart

Profile Plot



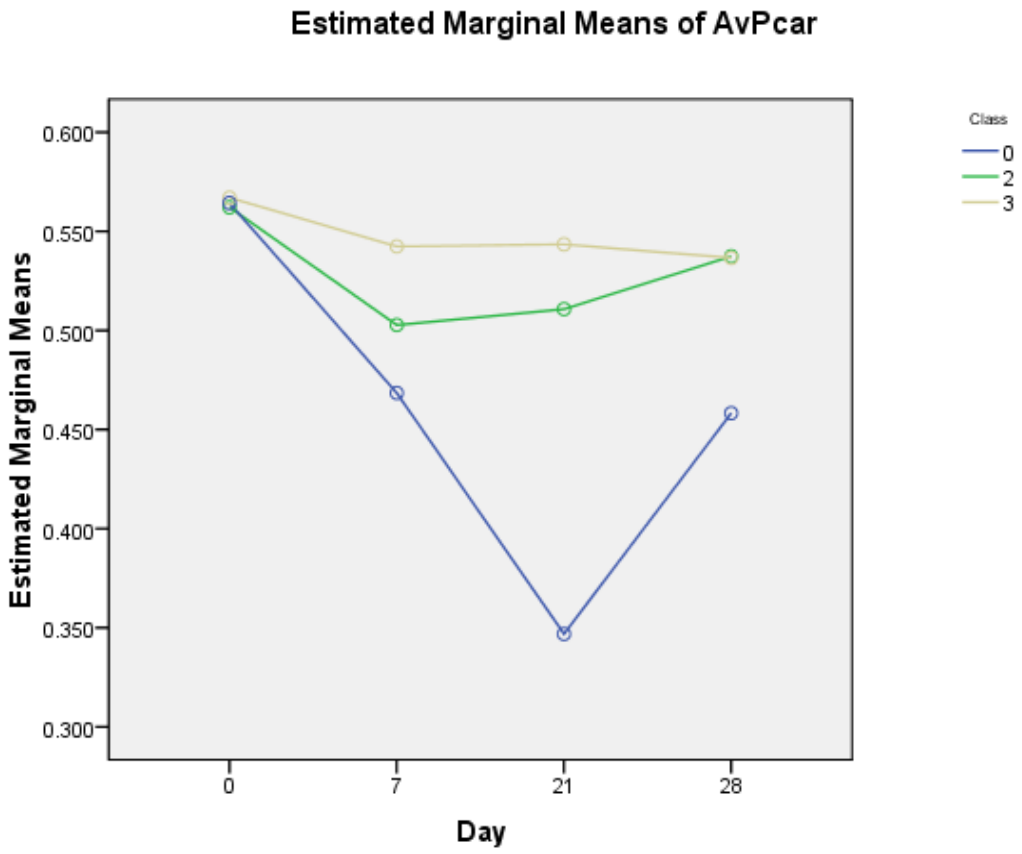
15.8.3 AvTvolP

Profile Plot



15.8.4 AvPcart

Profile Plot



15.8.5 Effects of Day on Class

15.8.5.1 AvTvol

15.8.5.1.1 Class 0

Between-Subjects Factors

		N
Day	0	9
	7	8
	21	10
	28	10

Tests of Between-Subjects Effects

Dependent Variable:AvTvol

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	10.061[a]	4	2.515	46.199	.000
Intercept	.006	1	.006	.116	.735
Tcube	3.701	1	3.701	67.982	.000
Day	1.101	3	.367	6.743	.001
Error	1.742	32	.054		
Total	230.947	37			
Corrected Total	11.803	36			

a. R Squared = .852 (Adjusted R Squared = .834)

Estimated Marginal Means

1. Grand Mean

Dependent Variable:AvTvol

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
2.444[a]	.039	2.366	2.523

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.25639.

2. Day

Estimates

Dependent Variable:AvTvol

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	2.766[a]	.089	2.585	2.947
7	2.475[a]	.082	2.307	2.643
21	2.294[a]	.076	2.139	2.450
28	2.241[a]	.076	2.086	2.396

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.25639.

Pairwise Comparisons

Dependent Variable:AvTvol

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.291	.121	.136	-.051	.632
	21	.471*	.124	.004	.122	.820
	28	.524*	.124	.001	.176	.873
7	0	-.291	.121	.136	-.632	.051
	21	.180	.112	.709	-.135	.496
	28	.233	.112	.273	-.082	.549
21	0	-.471*	.124	.004	-.820	-.122
	7	-.180	.112	.709	-.496	.135
	28	.053	.104	1.000	-.240	.347
28	0	-.524*	.124	.001	-.873	-.176
	7	-.233	.112	.273	-.549	.082
	21	-.053	.104	1.000	-.347	.240

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

*. The mean difference is significant at the .05 level.

Univariate Tests

Dependent Variable:AvTvol

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	1.101	3	.367	6.743	.001
Error	1.742	32	.054		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.8.5.1.2 Class 2

Between-Subjects Factors

		N
Day	0	10
	7	3
	21	4
	28	5

Tests of Between-Subjects Effects

Dependent Variable:AvTvol

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6.218[a]	4	1.554	15.935	.000
Intercept	.009	1	.009	.090	.768
Tcube	3.409	1	3.409	34.943	.000
Day	.900	3	.300	3.074	.056
Error	1.658	17	.098		
Total	176.709	22			
Corrected Total	7.876	21			

a. R Squared = .789 (Adjusted R Squared = .740)

Estimated Marginal Means

1. Grand Mean

Dependent Variable:AvTvol

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
2.734[a]	.076	2.573	2.895

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.68363.

2. Day

Estimates

Dependent Variable:AvTvol

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	2.933[a]	.103	2.715	3.151
7	2.809[a]	.218	2.350	3.269
21	2.776[a]	.157	2.445	3.108
28	2.415[a]	.142	2.115	2.716

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.68363.

Pairwise Comparisons

Dependent Variable:AvTvol

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.124	.256	1.000	-.640	.888
	21	.157	.191	1.000	-.412	.727
	28	.518*	.171	.046	.007	1.029
7	0	-.124	.256	1.000	-.888	.640
	21	.033	.261	1.000	-.744	.811
	28	.394	.273	.999	-.419	1.208
21	0	-.157	.191	1.000	-.727	.412
	7	-.033	.261	1.000	-.811	.744
	28	.361	.214	.662	-.278	1.000
28	0	-.518*	.171	.046	-1.029	-.007
	7	-.394	.273	.999	-1.208	.419
	21	-.361	.214	.662	-1.000	.278

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

*. The mean difference is significant at the .05 level.

Univariate Tests

Dependent Variable:AvTvol

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.900	3	.300	3.074	.056
Error	1.658	17	.098		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.8.5.1.3 Class 3

Between-Subjects Factors

		N
Day	0	8
	7	7
	21	6
	28	5

Tests of Between-Subjects Effects

Dependent Variable:AvTvol

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	10.323[a]	4	2.581	27.494	.000
Intercept	.088	1	.088	.942	.343
Tcube	4.146	1	4.146	44.168	.000
Day	.497	3	.166	1.765	.185
Error	1.971	21	.094		
Total	165.369	26			
Corrected Total	12.294	25			

a. R Squared = .840 (Adjusted R Squared = .809)

Estimated Marginal Means

1. Grand Mean

Dependent Variable:AvTvol

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
2.403[a]	.062	2.275	2.531

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 5.99778.

2. Day

Estimates

Dependent Variable:AvTvol

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	2.683[a]	.127	2.418	2.948
7	2.325[a]	.116	2.084	2.566
21	2.331[a]	.131	2.059	2.603
28	2.273[a]	.148	1.965	2.581

Estimates

Dependent Variable:AvTvol

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	2.683[a]	.127	2.418	2.948
7	2.325[a]	.116	2.084	2.566
21	2.331[a]	.131	2.059	2.603
28	2.273[a]	.148	1.965	2.581

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 5.99778.

Pairwise Comparisons

Dependent Variable:AvTvol

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.358	.174	.312	-.148	.864
	21	.352	.196	.527	-.220	.923
	28	.410	.214	.413	-.212	1.032
7	0	-.358	.174	.312	-.864	.148
	21	-.007	.174	1.000	-.513	.500
	28	.052	.187	1.000	-.492	.595
21	0	-.352	.196	.527	-.923	.220
	7	.007	.174	1.000	-.500	.513
	28	.058	.186	1.000	-.484	.601
28	0	-.410	.214	.413	-1.032	.212
	7	-.052	.187	1.000	-.595	.492
	21	-.058	.186	1.000	-.601	.484

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable:AvTvol

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.497	3	.166	1.765	.185
Error	1.971	21	.094		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.8.5.2 AvTcart

15.8.5.2.1 Class 0

Between-Subjects Factors

		N
Day	0	9
	7	8
	21	10
	28	10

Tests of Between-Subjects Effects

Dependent Variable:AvTcart

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.164[a]	4	.291	11.363	.000
Intercept	.123	1	.123	4.788	.036
Tcube	.162	1	.162	6.316	.017
Day	.392	3	.131	5.096	.005
Error	.820	32	.026		
Total	33.177	37			
Corrected Total	1.984	36			

a. R Squared = .587 (Adjusted R Squared = .535)

Estimated Marginal Means

1. Grand Mean

Dependent Variable:AvTcart

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
.923[a]	.026	.869	.977

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.25639.

2. Day

Estimates

Dependent Variable:AvTcart

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.118[a]	.061	.993	1.242
7	.913[a]	.057	.798	1.029
21	.787[a]	.052	.681	.894
28	.873[a]	.052	.767	.980

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.25639.

Pairwise Comparisons

Dependent Variable:AvTcart

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.204	.083	.119	-.030	.439
	21	.330*	.085	.003	.091	.569
	28	.244*	.085	.043	.005	.483
7	0	-.204	.083	.119	-.439	.030
	21	.126	.077	.673	-.091	.342
	28	.040	.077	1.000	-.177	.256
21	0	-.330*	.085	.003	-.569	-.091
	7	-.126	.077	.673	-.342	.091
	28	-.086	.072	1.000	-.287	.115
28	0	-.244*	.085	.043	-.483	-.005
	7	-.040	.077	1.000	-.256	.177
	21	.086	.072	1.000	-.115	.287

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

*. The mean difference is significant at the .05 level.

Univariate Tests

Dependent Variable:AvTcart

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.392	3	.131	5.096	.005
Error	.820	32	.026		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.8.5.2.2 Class 2

Between-Subjects Factors

		N
Day	0	10
	7	3
	21	4
	28	5

Tests of Between-Subjects Effects

Dependent Variable:AvTcart

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.938[a]	4	.234	19.675	.000
Intercept	.000	1	.000	.013	.911
Tcube	.522	1	.522	43.800	.000
Day	.087	3	.029	2.420	.102
Error	.203	17	.012		
Total	25.502	22			
Corrected Total	1.140	21			

a. R Squared = .822 (Adjusted R Squared = .781)

Estimated Marginal Means

1. Grand Mean

Dependent Variable:AvTcart

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
1.037[a]	.027	.980	1.093

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.68363.

2. Day

Estimates

Dependent Variable:AvTcart

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.111[a]	.036	1.035	1.188
7	1.077[a]	.076	.917	1.238
21	.984[a]	.055	.868	1.100
28	.973[a]	.050	.869	1.078

Estimates

Dependent Variable:AvTcart

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.111[a]	.036	1.035	1.188
7	1.077[a]	.076	.917	1.238
21	.984[a]	.055	.868	1.100
28	.973[a]	.050	.869	1.078

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.68363.

Pairwise Comparisons

Dependent Variable:AvTcart

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.034	.090	1.000	-.233	.301
	21	.127	.067	.441	-.072	.326
	28	.138	.060	.203	-.040	.317
7	0	-.034	.090	1.000	-.301	.233
	21	.093	.091	1.000	-.179	.365
	28	.104	.095	1.000	-.180	.388
21	0	-.127	.067	.441	-.326	.072
	7	-.093	.091	1.000	-.365	.179
	28	.011	.075	1.000	-.213	.234
28	0	-.138	.060	.203	-.317	.040
	7	-.104	.095	1.000	-.388	.180
	21	-.011	.075	1.000	-.234	.213

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable:AvTcart

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.087	3	.029	2.420	.102
Error	.203	17	.012		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.8.5.2.3 Class 3

Between-Subjects Factors

		N
Day	0	8
	7	7
	21	6
	28	5

Tests of Between-Subjects Effects

Dependent Variable:AvTcart

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.879[a]	4	.220	14.223	.000
Intercept	.119	1	.119	7.680	.011
Tcube	.400	1	.400	25.891	.000
Day	.025	3	.008	.529	.667
Error	.324	21	.015		
Total	27.686	26			
Corrected Total	1.203	25			

a. R Squared = .730 (Adjusted R Squared = .679)

Estimated Marginal Means

1. Grand Mean

Dependent Variable:AvTcart

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
1.005[a]	.025	.953	1.056

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 5.99778.

2. Day

Estimates

Dependent Variable:AvTcart

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.065[a]	.052	.958	1.173
7	.989[a]	.047	.892	1.087
21	.975[a]	.053	.864	1.085
28	.989[a]	.060	.864	1.114

Estimates

Dependent Variable:AvTcart

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.065[a]	.052	.958	1.173
7	.989[a]	.047	.892	1.087
21	.975[a]	.053	.864	1.085
28	.989[a]	.060	.864	1.114

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 5.99778.

Pairwise Comparisons

Dependent Variable:AvTcart

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.076	.071	1.000	-.129	.281
	21	.091	.080	1.000	-.141	.323
	28	.076	.087	1.000	-.176	.329
7	0	-.076	.071	1.000	-.281	.129
	21	.015	.071	1.000	-.191	.220
	28	.000	.076	1.000	-.220	.221
21	0	-.091	.080	1.000	-.323	.141
	7	-.015	.071	1.000	-.220	.191
	28	-.014	.076	1.000	-.234	.206
28	0	-.076	.087	1.000	-.329	.176
	7	.000	.076	1.000	-.221	.220
	21	.014	.076	1.000	-.206	.234

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable:AvTcart

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.025	3	.008	.529	.667
Error	.324	21	.015		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.8.5.3 AvTvolP

15.8.5.3.1 Class 0

Between-Subjects Factors

		N
Day	0	9
	7	8
	21	10
	28	10

Tests of Between-Subjects Effects

Dependent Variable:AvTvolP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.770[a]	4	.943	15.454	.000
Intercept	.093	1	.093	1.524	.226
Tcube	1.753	1	1.753	28.741	.000
Day	.222	3	.074	1.212	.321
Error	1.952	32	.061		
Total	61.985	37			
Corrected Total	5.722	36			

a. R Squared = .659 (Adjusted R Squared = .616)

Estimated Marginal Means

1. Grand Mean

Dependent Variable:AvTvolP

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
1.238[a]	.041	1.155	1.321

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.25639.

2. Day

Estimates

Dependent Variable:AvTvolP

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.374[a]	.094	1.182	1.566
7	1.257[a]	.087	1.079	1.435
21	1.129[a]	.081	.965	1.294
28	1.191[a]	.081	1.027	1.355

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.25639.

Pairwise Comparisons

Dependent Variable:AvTvolP

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.117	.129	1.000	-.244	.479
	21	.245	.131	.430	-.125	.614
	28	.183	.131	1.000	-.185	.552
7	0	-.117	.129	1.000	-.479	.244
	21	.128	.119	1.000	-.207	.462
	28	.066	.119	1.000	-.268	.400
21	0	-.245	.131	.430	-.614	.125
	7	-.128	.119	1.000	-.462	.207
	28	-.061	.110	1.000	-.372	.249
28	0	-.183	.131	1.000	-.552	.185
	7	-.066	.119	1.000	-.400	.268
	21	.061	.110	1.000	-.249	.372

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable:AvTvolP

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.222	3	.074	1.212	.321
Error	1.952	32	.061		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.8.5.3.2 Class 2

Between-Subjects Factors

		N
Day	0	10
	7	3
	21	4
	28	5

Tests of Between-Subjects Effects

Dependent Variable:AvTvolP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.338[a]	4	.584	7.050	.002
Intercept	.063	1	.063	.756	.397
Tcube	1.625	1	1.625	19.603	.000
Day	.136	3	.045	.545	.658
Error	1.409	17	.083		
Total	50.235	22			
Corrected Total	3.747	21			

a. R Squared = .624 (Adjusted R Squared = .535)

Estimated Marginal Means

1. Grand Mean

Dependent Variable:AvTvolP

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
1.457[a]	.070	1.309	1.605

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.68363.

2. Day

Estimates

Dependent Variable:AvTvolP

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.487[a]	.095	1.286	1.688
7	1.569[a]	.201	1.145	1.993
21	1.461[a]	.145	1.156	1.767
28	1.311[a]	.131	1.035	1.588

Estimates

Dependent Variable:AvTvolP

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.487[a]	.095	1.286	1.688
7	1.569[a]	.201	1.145	1.993
21	1.461[a]	.145	1.156	1.767
28	1.311[a]	.131	1.035	1.588

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.68363.

Pairwise Comparisons

Dependent Variable:AvTvolP

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	-.082	.236	1.000	-.786	.623
	21	.026	.176	1.000	-.499	.551
	28	.176	.158	1.000	-.295	.647
7	0	.082	.236	1.000	-.623	.786
	21	.108	.240	1.000	-.609	.824
	28	.258	.251	1.000	-.492	1.008
21	0	-.026	.176	1.000	-.551	.499
	7	-.108	.240	1.000	-.824	.609
	28	.150	.197	1.000	-.439	.739
28	0	-.176	.158	1.000	-.647	.295
	7	-.258	.251	1.000	-1.008	.492
	21	-.150	.197	1.000	-.739	.439

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable:AvTvolP

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.136	3	.045	.545	.658
Error	1.409	17	.083		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.8.5.3.3 Class 3

Between-Subjects Factors

		N
Day	0	8
	7	7
	21	6
	28	5

Tests of Between-Subjects Effects

Dependent Variable:AvTvolP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.862[a]	4	.965	11.868	.000
Intercept	5.866E-5	1	5.866E-5	.001	.979
Tcube	1.287	1	1.287	15.825	.001
Day	.306	3	.102	1.253	.316
Error	1.708	21	.081		
Total	42.936	26			
Corrected Total	5.570	25			

a. R Squared = .693 (Adjusted R Squared = .635)

Estimated Marginal Means

1. Grand Mean

Dependent Variable:AvTvolP

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
1.177[a]	.057	1.058	1.296

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 5.99778.

2. Day

Estimates

Dependent Variable:AvTvolP

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.378[a]	.119	1.131	1.625
7	1.222[a]	.108	.997	1.446
21	1.052[a]	.122	.798	1.305
28	1.057[a]	.138	.771	1.343

Estimates

Dependent Variable:AvTvolP

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	1.378[a]	.119	1.131	1.625
7	1.222[a]	.108	.997	1.446
21	1.052[a]	.122	.798	1.305
28	1.057[a]	.138	.771	1.343

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 5.99778.

Pairwise Comparisons

Dependent Variable:AvTvolP

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.156	.162	1.000	-.315	.628
	21	.326	.183	.532	-.206	.858
	28	.321	.199	.728	-.258	.900
7	0	-.156	.162	1.000	-.628	.315
	21	.170	.162	1.000	-.301	.641
	28	.165	.174	1.000	-.341	.671
21	0	-.326	.183	.532	-.858	.206
	7	-.170	.162	1.000	-.641	.301
	28	-.005	.173	1.000	-.510	.500
28	0	-.321	.199	.728	-.900	.258
	7	-.165	.174	1.000	-.671	.341
	21	.005	.173	1.000	-.500	.510

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable:AvTvolP

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.306	3	.102	1.253	.316
Error	1.708	21	.081		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.8.5.4 AvPcart

15.8.5.4.1 Class 0

Between-Subjects Factors

		N
Day	0	9
	7	8
	21	10
	28	10

Tests of Between-Subjects Effects

Dependent Variable:AvPcar

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.526[a]	4	.131	6.109	.001
Intercept	.018	1	.018	.852	.363
Tcube	.057	1	.057	2.642	.114
Day	.222	3	.074	3.439	.028
Error	.688	32	.022		
Total	8.840	37			
Corrected Total	1.214	36			

a. R Squared = .433 (Adjusted R Squared = .362)

Estimated Marginal Means

1. Grand Mean

Dependent Variable:AvPcar

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
.458[a]	.024	.408	.507

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.25639.

2. Day

Estimates

Dependent Variable:AvPcar

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	.583[a]	.056	.469	.697
7	.466[a]	.052	.360	.572
21	.335[a]	.048	.238	.433
28	.447[a]	.048	.350	.545

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.25639.

Pairwise Comparisons

Dependent Variable:AvPcar

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.117	.076	.813	-.098	.332
	21	.248*	.078	.020	.028	.467
	28	.136	.078	.541	-.083	.355
7	0	-.117	.076	.813	-.332	.098
	21	.131	.071	.439	-.068	.329
	28	.019	.071	1.000	-.179	.217
21	0	-.248*	.078	.020	-.467	-.028
	7	-.131	.071	.439	-.329	.068
	28	-.112	.066	.589	-.296	.073
28	0	-.136	.078	.541	-.355	.083
	7	-.019	.071	1.000	-.217	.179
	21	.112	.066	.589	-.073	.296

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

*. The mean difference is significant at the .05 level.

Univariate Tests

Dependent Variable:AvPcar

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.222	3	.074	3.439	.028
Error	.688	32	.022		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.8.5.4.2 Class 2

Between-Subjects Factors

		N
Day	0	10
	7	3
	21	4
	28	5

Tests of Between-Subjects Effects

Dependent Variable:AvPcar

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.422[a]	4	.105	9.027	.000
Intercept	.022	1	.022	1.887	.187
Tcube	.300	1	.300	25.669	.000
Day	.008	3	.003	.242	.866
Error	.199	17	.012		
Total	7.672	22			
Corrected Total	.620	21			

a. R Squared = .680 (Adjusted R Squared = .605)

Estimated Marginal Means

1. Grand Mean

Dependent Variable:AvPcar

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
.570[a]	.026	.514	.626

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.68363.

2. Day

Estimates

Dependent Variable:AvPcar

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	.568[a]	.036	.492	.643
7	.617[a]	.075	.458	.777
21	.550[a]	.054	.436	.665
28	.545[a]	.049	.441	.649

Estimates

Dependent Variable:AvPcar

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	.568[a]	.036	.492	.643
7	.617[a]	.075	.458	.777
21	.550[a]	.054	.436	.665
28	.545[a]	.049	.441	.649

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 6.68363.

Pairwise Comparisons

Dependent Variable:AvPcar

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	-.050	.089	1.000	-.314	.215
	21	.017	.066	1.000	-.180	.214
	28	.022	.059	1.000	-.154	.199
7	0	.050	.089	1.000	-.215	.314
	21	.067	.090	1.000	-.202	.336
	28	.072	.094	1.000	-.209	.354
21	0	-.017	.066	1.000	-.214	.180
	7	-.067	.090	1.000	-.336	.202
	28	.005	.074	1.000	-.216	.226
28	0	-.022	.059	1.000	-.199	.154
	7	-.072	.094	1.000	-.354	.209
	21	-.005	.074	1.000	-.226	.216

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable:AvPcar

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.008	3	.003	.242	.866
Error	.199	17	.012		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.8.5.4.3 Class 3

Between-Subjects Factors

		N
Day	0	8
	7	7
	21	6
	28	5

Tests of Between-Subjects Effects

Dependent Variable:AvPcar

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.244[a]	4	.061	3.559	.023
Intercept	.038	1	.038	2.218	.151
Tcube	.100	1	.100	5.823	.025
Day	.010	3	.003	.192	.900
Error	.360	21	.017		
Total	7.886	26			
Corrected Total	.604	25			

a. R Squared = .404 (Adjusted R Squared = .291)

Estimated Marginal Means

1. Grand Mean

Dependent Variable:AvPcar

Mean	Std. Error	95% Confidence Interval	
		Lower Bound	Upper Bound
.525[a]	.026	.471	.580

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 5.99778.

2. Day

Estimates

Dependent Variable:AvPcar

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	.565[a]	.054	.452	.678
7	.521[a]	.050	.418	.624
21	.513[a]	.056	.397	.630
28	.502[a]	.063	.370	.633

Estimates

Dependent Variable:AvPcar

Day	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
0	.565[a]	.054	.452	.678
7	.521[a]	.050	.418	.624
21	.513[a]	.056	.397	.630
28	.502[a]	.063	.370	.633

a. Covariates appearing in the model are evaluated at the following values: Total cube (mm) = 5.99778.

Pairwise Comparisons

Dependent Variable:AvPcar

(I) Day	(J) Day	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for Difference[a]	
					Lower Bound	Upper Bound
0	7	.044	.074	1.000	-.173	.260
	21	.052	.084	1.000	-.192	.296
	28	.063	.091	1.000	-.202	.329
7	0	-.044	.074	1.000	-.260	.173
	21	.008	.074	1.000	-.208	.225
	28	.020	.080	1.000	-.213	.252
21	0	-.052	.084	1.000	-.296	.192
	7	-.008	.074	1.000	-.225	.208
	28	.011	.080	1.000	-.220	.243
28	0	-.063	.091	1.000	-.329	.202
	7	-.020	.080	1.000	-.252	.213
	21	-.011	.080	1.000	-.243	.220

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Univariate Tests

Dependent Variable:AvPcar

	Sum of Squares	df	Mean Square	F	Sig.
Contrast	.010	3	.003	.192	.900
Error	.360	21	.017		

The F tests the effect of Day. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

15.9Surgical spreadsheet

Rat ID no.	Weight	Arrival Date	Procedure Date	Sacrifice Date	Procedure Performed/Group	Time Point	Weight @ removal	Weight @ sacrifice	Comments	Ear tag
GLD001	NA	7/08/2007	NA	21/08/2007	Group 1	Day 0	NA	51.7	Baseline Controls	NA
GLD002	NA	7/08/2007	NA	21/08/2007	Group 1	Day 0	NA	49.1	Baseline Controls	NA
GLD003	NA	7/08/2007	NA	21/08/2007	Group 1	Day 0	NA	48.4	Baseline Controls	NA
GLD004	NA	7/08/2007	NA	21/08/2007	Group 1	Day 0	NA	47.8	Baseline Controls	NA
GLD005	NA	7/08/2007	NA	21/08/2007	Group 1	Day 0	NA	43.9	Baseline Controls	NA
GLD006	NA	7/08/2007	NA	28/08/2007	Group 1	Day 7	NA	45.7		NA
GLD007	NA	7/08/2007	NA	28/08/2007	Group 1	Day 7	NA	54.2		NA
GLD008	NA	7/08/2007	NA	28/08/2007	Group 1	Day 7	NA	45.1		NA
GLD009	NA	7/08/2007	NA	28/08/2007	Group 1	Day 7	NA	44.0		NA
GLD010	NA	7/08/2007	NA	28/08/2007	Group 1	Day 7	NA	46.8		NA
GLD011	NA	7/08/2007	NA	18/09/2007	Group 1	Day 30	NA	45.9		NA
GLD012	NA	7/08/2007	NA	18/09/2007	Group 1	Day 30	NA	43.2		NA
GLD013	NA	7/08/2007	NA	11/09/2007	Group 1	Day 21	NA	44.8		NA
GLD014	NA	7/08/2007	NA	11/09/2007	Group 1	Day 21	NA	45.6		NA
GLD015	NA	7/08/2007	NA	11/09/2007	Group 1	Day 21	NA	47.7		NA
GLD016	NA	7/08/2007	NA	11/09/2007	Group 1	Day 21	NA	47.6		NA
GLD017	NA	7/08/2007	NA	11/09/2007	Group 1	Day 21	NA	45.9		NA
GLD018	NA	7/08/2007	NA	18/09/2007	Group 1	Day 30	NA	38.6		NA
GLD019	NA	7/08/2007	NA	18/09/2007	Group 1	Day 30	NA	42.3		NA
GLD020	NA	7/08/2007	NA	18/09/2007	Group 1	Day 30	NA	48.8		NA
GLD021	39.6	7/08/2007	21/08/2007	28/08/2007	Group 2	Day 7	NA	33.4		None
GLD022	36.4	7/08/2007	21/08/2007	28/08/2007	Group 2	Day 7	NA	33.3		1L
GLD023	43.9	7/08/2007	21/08/2007	28/08/2007	Group 2	Day 7	NA	41.1		1R
GLD024	46.6	7/08/2007	21/08/2007	28/08/2007	Group 2	Day 7	NA	41.1		Tail
GLD025	44.6	7/08/2007	21/08/2007	18/09/2007	Group 2	Day 30	39.3	39.6		None
GLD026	46.2	7/08/2007	21/08/2007	18/09/2007	Group 2	Day 30	39.2	37.3		1L

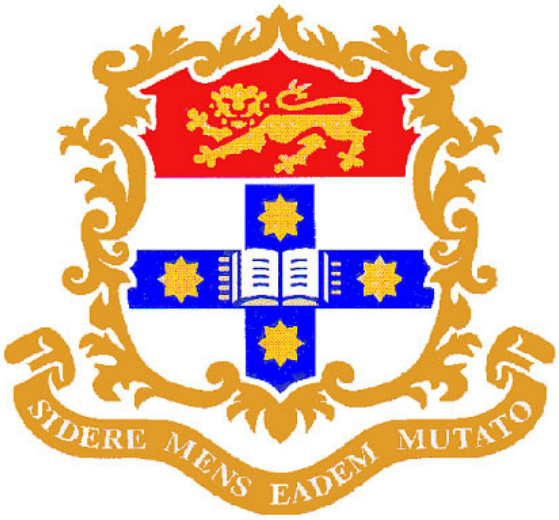
Rat ID no.	Weight	Arrival Date	Procedure Date	Sacrifice Date	Procedure Performed/Group	Time Point	Weight @ removal	Weight @ sacrifice	Comments	Ear tag
GLD027	41.3	7/08/2007	21/08/2007	11/09/2007	Group 2	Day 21	NA	35.9		1R
GLD028	45.2	7/08/2007	21/08/2007	18/09/2007	Group 2	Day 30	37.8	36.9		Tail
GLD029	44.5	7/08/2007	21/08/2007	18/09/2007	Group 2	Day 30	37.2	35.7		None
GLD030	39.9	7/08/2007	21/08/2007	28/08/2007	Group 2	Day 7	NA	36.1		1L
GLD031	43.2	7/08/2007	21/08/2007	18/09/2007	Group 2	Day 30	37.8	37.0		1R
GLD032	37.3	7/08/2007	21/08/2007	11/09/2007	Group 2	Day 21	NA	31.5		Tail
GLD033	40.5	7/08/2007	21/08/2007	11/09/2007	Group 2	Day 21	NA	36.5		None
GLD034	50.4	7/08/2007	21/08/2007	11/09/2007	Group 2	Day 21	NA	43.2		1L
GLD035	44.1	7/08/2007	21/08/2007	11/09/2007	Group 2	Day 21	NA	38.3		1R

Rat ID no.	Weight	Arrival Date	Procedure Date	Sacrifice Date	Procedure Performed/Group	Time Point	Weight @ removal	Weight @ sacrifice	Comments	Ear tag
GLD071	NA	9/08/2007	NA	23/08/2007	Group 1	Day 0	NA	44.8	Baseline Controls	NA
GLD072	NA	9/08/2007	NA	23/08/2007	Group 1	Day 0	NA	46.2	Baseline Controls	NA
GLD073	NA	9/08/2007	NA	23/08/2007	Group 1	Day 0	NA	42.2	Baseline Controls	NA
GLD074	NA	9/08/2007	NA	23/08/2007	Group 1	Day 0	NA	42.8	Baseline Controls	NA
GLD075	NA	9/08/2007	NA	23/08/2007	Group 1	Day 0	NA	41.8	Baseline Controls	NA
GLD076	NA	9/08/2007	NA	30/08/2007	Group 1	Day 7	NA	45.9		NA
GLD077	NA	9/08/2007	NA	30/08/2007	Group 1	Day 7	NA	44.2		NA
GLD078	NA	9/08/2007	NA	30/08/2007	Group 1	Day 7	NA	40.9		NA
GLD079	NA	9/08/2007	NA	30/08/2007	Group 1	Day 7	NA	42.2		NA
GLD080	NA	9/08/2007	NA	30/08/2007	Group 1	Day 7	NA	41.5		NA
GLD081	NA	9/08/2007	NA	13/09/2007	Group 1	Day 21	NA	40.3		NA
GLD082	NA	9/08/2007	NA	13/09/2007	Group 1	Day 21	NA	41.4		NA
GLD083	NA	9/08/2007	NA	13/09/2007	Group 1	Day 21	NA	37.8		NA
GLD084	NA	9/08/2007	NA	20/09/2007	Group 1	Day 28	NA	42.7		NA
GLD085	NA	9/08/2007	NA	20/09/2007	Group 1	Day 28	NA	40.0		NA
GLD086	NA	9/08/2007	NA	20/09/2007	Group 1	Day 28	NA	49.5		NA
GLD087	NA	9/08/2007	NA	13/09/2007	Group 1	Day 21	NA	36.1		NA
GLD088	NA	9/08/2007	NA	13/09/2007	Group 1	Day 21	NA	37.8		NA

Rat ID no.	Weight	Arrival Date	Procedure Date	Sacrifice Date	Procedure Performed/Group	Time Point	Weight @ removal	Weight @ sacrifice	Comments	Ear tag
GLD089	NA	9/08/2007	NA	20/09/2007	Group 1	Day 28	NA	38.9		NA
GLD090	NA	9/08/2007	NA	20/09/2007	Group 1	Day 28	NA	37.6		NA
GLD091	40.1	9/08/2007	23/08/2007	30/08/2007	Group 2	Day 7	NA	39.2		None
GLD092	41.9	9/08/2007	23/08/2007	30/08/2007	Group 2	Day 7	NA	39.8		1L
GLD093	40.6	9/08/2007	23/08/2007	20/09/2007	Group 2	Day 28	35.6	38.4		1R
GLD094	38.3	9/08/2007	23/08/2007	20/09/2007	Group 2	Day 28	34.7	40.3		Tail
GLD095	31.8	9/08/2007	23/08/2007	30/08/2007	Group 2	Day 7	NA	27.7		None
GLD096	36.1	9/08/2007	23/08/2007	20/09/2007	Group 2	Day 28	32.0	37.2		1L
GLD097	32.9	9/08/2007	23/08/2007	20/09/2007	Group 2	Day 28	26.9	31.8		1R
GLD098	39.6	9/08/2007	23/08/2007	30/08/2007	Group 2	Day 7	NA	36.3		Tail
GLD099	38.8	9/08/2007	23/08/2007	20/09/2007	Group 2	Day 28	36.1	41.9		None
GLD100	40.5	9/08/2007	23/08/2007	13/09/2007	Group 2	Day 21	NA	33.4		1L
GLD101	39.4	9/08/2007	23/08/2007	13/09/2007	Group 2	Day 21	NA	34.4		1R
GLD102	33.5	9/08/2007	23/08/2007	13/09/2007	Group 2	Day 21	NA	26.1		Tail
GLD103	41.3	9/08/2007	23/08/2007	13/09/2007	Group 2	Day 21	NA	34.4		None
GLD104	40.7	9/08/2007	23/08/2007	30/08/2007	Group 2	Day 7	NA	39.2		1L
GLD105	36.9	9/08/2007	23/08/2007	13/09/2007	Group 2	Day 21	NA	31.4		1R

16 Manuscript

Effect of Mandibular Displacement on Condylar Cartilage Remodeling in Sprague Dawley Rats: A Micro-Structural Analysis



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Effect of Mandibular Displacement on Condylar Cartilage Remodelling in Sprague Dawley Rats: A Micro-Structural Analysis

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ABSTRACT:

Recent advances in imaging techniques allowed enhancement of cartilage visualisation for Micro-CT analysis. This provided a new method for analysing both qualitative and quantitative changes in the condylar cartilage

Aim: The objective of this study was to provide a new insight in understanding changes in condylar cartilage with normal growth and after the placement of a functional appliance over a four-week-period.

Materials and Methods: Bite ramps were placed on the lower incisors of 30 Sprague Dawley Rats to induce mandibular posture. The rats were sacrificed at Day 0, 7, 21, 28 with corresponding control group (n=40). Specimens were stained in Gadolinium Chloride and scanned via MicroCT. Condylar cartilage was then digitally extracted for qualitative and quantitative analysis. Repeated measurements were used to evaluate the method error of this innovative technique.

Results: Morphologically changes were noted in the shape of the condyle between groups and over the treatment duration. Significant remodelling and distinctive irregular shape were noted in anteriorly postured mandibles. Quantitative analysis demonstrated differences between control and appliance groups. Method error analysis highlighted the protocol's consistency in volumetric measurement.

Discussion: This study demonstrated a new three dimensional method of analysing changes in the condylar cartilage following orthopaedic intervention, further improving our understanding of the growth of the condyle and its manipulation.

INTRODUCTION:

Functional appliances are commonly used to exploit the potential for enhanced condylar cartilage growth conducive to the correction of mandibular retrusion in orthodontic patients. This articular cartilage is adaptive to functional demands and animal studies have demonstrated the potential for mandibular growth beyond that expected of normal growth [1-4]. It has also been shown to adaptively change when subjected to compressive forces via appliances [5,6] and changes in occlusal function [7,8]. Changes in the condylar cartilage have been depicted mainly via cephalometric or histological methods [1,7,9-13]. Limited studies [14] have been carried out via MicroCT on cartilage due to its inherent weak attenuation. Recent advances in MRI staining medium have been successfully applied to MicroCT to improve cartilage visualisation [15]. The objective of this study was to apply recent advances in imaging technology to enhance our understanding of changes in condylar cartilage with normal growth and after the placement of a functional appliance over a four-week-period in Sprague Dawley rats.

MATERIALS AND METHODS

Seventy Sprague Dawley (five-week-old) rats were received. The animal model, type and experimentation protocol replicated previous studies in this area [16,17]. Ten were sacrificed for baseline assessment and the remaining 60 rats randomly divided into control and experimental groups. Animals were fed a restricted diet of Nutrigel (Troy Laboratories, Australia) for comparisons to animals given growth supplements in a concurrent study [18]. Composite based bite ramps (Upper paediatric incisor crown formers; Z100 Dental

composite, 3M ESPE, USA) were attached to the lower incisors of the experimental animals under inhalational isofluorane anaesthesia. Ten experimental and 10 control animals were subsequently sacrificed at Days 7, 21, and 28. Day 28 animals had appliances removed at Day 21 to assess changes between Days 21 to 28. All animals were assessed daily by investigators and animal housing staff for signs of distress. At sacrifice, right hemisections were carefully degloved of soft tissue, maintaining the ligamentous structure of the temporomandibular joint before being stored in 10% buffered formalin individually. Specimens were then placed in 200mM Gadolinium Chloride solution [15] for six days (± 12hours) for optimal diffusion of staining medium. Ethics approval was obtained from the University of New South Wales (Authority to Conduct Animal Research Project (ACEC # 07/31B).

Imaging Analysis

Specimens were scanned in a SkyScan 1172 MicroCT (Skyscan, Belgium) scanner at 7um resolution with 0.39 degree rotational increments before being reconstructed. Specifically designed software (RotateScanline, Electron Microscopy Unit, University of Sydney) was used to reorientate samples into a standardised orientation to reflect their anatomical angulation. Samples were aligned so that the condylar axis of the condylar head was 15 degrees to the mid-sagittal plane with the lateral poles of the condyle parallel to a reference axis. Once orientated, VGStudioMax (V1.2 - 64 Bit version, Volume Graphics, Germany) was used to digitally extract the condylar head from the surrounding hard and soft tissues. A standardised protocol of appropriate grayscale selection and standardised volumetric expansion of the selected area was followed in all

measurements. Volumetric measurements were taken of the whole condylar head (cartilage, bone and periosteum - CBP). The cartilage and periosteum (CP) was then digitally extracted and measured volumetrically. Volumetric measurements were also taken of the total posterior hemisection CBP and the posterior hemisection CP. A total cube (Tcube) covariable was developed from the product of the maximum linear dimensions of the condylar head to compensate for size differences between animals. The volumes for all parameters were remeasured for method error analysis. Univariate Analysis of Variance via a statistical program (SPSS V16.0, USA) was used to analyse the changes in the Total CBP volume of the condylar head (AvTvol), Total volume of the posterior CBP hemisection of the condylar head (AvTvolP), Cartilage CP volume of the condylar head (AvTcart) and Cartilage volume of the posterior hemisection of the condylar head (AvPCart). The data was then analysed for the differences between groups over the experimental period. Three dimensional images were also taken for gross morphologic investigations.

RESULTS:

Analysis was carried out on 67 of the 70 specimens as three samples demonstrated difficulties in the imaging extraction technique. No animal exhibited signs of distress; however no significant weight gain was noted in the control animals and significant weight loss occurred in experimental animals over the duration of the experiment. Due to the anatomy of the animals and the differing patterns of attrition of the appliances between individual animals, the experimental group demonstrated two jaw postures. An anteriorly postured (FWD – Class 3 group) position occurred as the bite ramp guided the

mandible forward and the condyle away from its articulation. A posteriorly postured (BWD – Class 2 group) position occurred when the bite ramp prevented forward posturing as it contacted the lingual surface of the upper incisors, resulting in posteriorly directed pressure on the temporomandibular joint. A Control group (Class 0) was used for comparisons. Table 1 illustrates the quantitative changes in each group over the 28-day period.

Quantitative Analysis:

1. Effects of Time:

There was a highly significant ($p<0.01$) reduction in the AvTvol, AvTcart and AvTvolP over the experimental period (Table 1). With AvPcart, this reduction was only marginally significant ($p<0.05$). Pairwise comparison of these dependent variables highlighted significant reductions in AvTvol, AvTcart and AvPcart between Day 0 and Days 7, 21, or 28 ($p<0.005$). There was no significant difference between Days 7, 21 and 28. AvTvolP demonstrated marginal significant ($p<0.05$) differences only between Day 0 and Days 21 or 28.

2. Effects of Appliance:

The presence of an appliance, resulting in anterior or posterior mandibular displacement, illustrated a highly significant ($p<0.001$) difference detected in the AvTcart and AvPcart. Pairwise comparison of these dependent variables illustrated a highly significant difference between Control and FWD groups ($p<0.005$).

An assessment using a Bonferroni adjustment for multiple tests was also made of the effects following appliance removal seven days prior; however no significant difference was noted between any of the dependent variables between Day 21 and Day 28.

Though there were no significant differences seen between BWD and FWD groups, there was an inverse trend visible in all dependent variables. With time, the AvTvol (Figure 3) and AvTvolP of the Control and FWD groups demonstrated a steady decline over time, while the BWD group demonstrated a slight increase over the same time period. However, in regards to AvPcart and AvTcart, Control groups demonstrated a slight increase in cartilage volumes between Day 21 and 28, similar to the steady increase of the BWD groups from Day 7 to 28. FWD groups demonstrated a consistent reduction in volume through to Day 28.

Qualitative Analysis:

Qualitative analysis demonstrated a general conformation shape change from a tear drop (posteriorly weighted) shape to a symmetrical (centrally weighted) ovoid shape in all groups over time. Significant remodelling was noted in the FWD group, with evidence of loss of general shape and curvature of structures with time (Figure 1). Additionally, there

was the development of obvious notching on the posterior border of the condylar head (Figure 2).

Over the duration of the experiment, no change in anteroposterior length of the condylar head was noted in any group. However, changes were noted in the superoinferior and mediolateral dimensions over time between Class groups. The Control group demonstrated a significant reduction in size of the condyle in both superoinferior and mediolateral dimensions over time. This reduction in superoinferior dimension was also seen in BWD and FWD groups. However, unlike Control and FWD groups, BWD groups demonstrated a non significant change in mediolateral dimension over time.

When examining all three groups together, no significant difference was noted in the mediolateral dimension over the experimental period. Anteroposterior dimension was significantly different between Day 0, 7, 21 and 28. There was a significant interaction present between the groups over the experimental period ($p<0.05$).

A method error analysis was also conducted to assess the consistency of results (Table 2) via a paired T-test. The coefficient of variation of measurements ranged from 5-13%.

DISCUSSION:

MicroCT has been effective in the *in vitro* inspection of bones in small animals [19]. However imaging of cartilage has always been difficult due to its inherently low contrast

[15]. Advances in magnetic resonance imaging (MRI) have seen the application of gadolinium based staining mediums filtrating into MicroCT use and has recently been demonstrated successfully [15,20].

The objective of this study was to develop a protocol in which the condylar cartilage of the mandibular condyle of the Sprague Dawley Rat could be visualised. Subsequently, this was the first investigation to three dimensionally visualise, with a specific staining medium and MicroCT, condylar cartilage at high resolution (7um). This allowed the accurate, non destructive assessment of changes due to growth and functional intervention to be measured qualitatively and quantitatively.

A previously developed protocol by Cockman et al [15] was adopted for use in orthodontic applications. After preliminary trials, it was determined 6 days ± 12 hours provided the best staining requirements with200mM Gadolinium Chloride. Unlike bovine nasal cartilage disks directly immersed in solution for 24 hours [15], this investigation required the same concentration of solution to diffuse through surrounding musculoskeletal and ligamentous tissues associated with the undisturbed tempormandibular joint. A standardised protocol was developed to enable the bone/cartilage/periosteal (CBP) and cartilage/periosteal (CP) volumes to be calculated accurately and reproducibly with a method of error between 5-13%. Though the measurements did not depict absolute volumes of CP or CBP, this protocol provided a standardised method, in which all samples were measured identically to allow comparative measurements to be made.

This study highlights the need to further investigate the effects of altered condylar function. Traditionally, two dimensional studies have been used to assess changes that occurred in the condylar cartilage as a result of altered function, growth and therapeutic intervention. These studies primarily consisted of radiological studies [1,7,11,21] or histological studies [1,9,11-13]. Recently, interest in the use of growth factors in modulating the growth of the condylar cartilage has resulted in the increased use of biochemical and immunohistochemical studies [17,22-27]. Hence, any changes in the volume of the cartilage have been depicted as either a gross morphological study or inferred from two dimensional conclusions based on cartilage thickness, altered expression of cellular layers or the use of immunohistochemical markers [7,8,12] . There are limited studies examining cartilage changes that occurred three dimensionally, with these studies typically either animal studies involving MRI or clinical studies with the use of CBCT [28-32] . However, these are limited by resolution and clarity of images as well as sample size. MicroCT studies have been limited to bony changes [33]; however this study successfully demonstrates its use in identifying cartilage changes.

A significant reduction in CBP and CP for the whole condyle and the posterior hemisection was demonstrated, consistent with the failure of any group to gain substantial weight during their pubertal growth spurt. Though there is limited literature regarding the weight gain of experimental animals, this study was consistent with a previous study [9] which demonstrated similar weight loss during experimentation. Orthodontic functional appliances have been implicated in the adaptability of condylar cartilage particularly when treatment is appropriated at the pubertal growth spurt [1,4-6]. The

failure of the animals to gain substantial weight in this study could be attributed to the appliance placement and the restrictive soft diet. This could have influenced the growth of the animal but also reduced the functional loading of the condylar head. Previous studies [7,8,34] have demonstrated the reduction in volume of the condylar head associated with reduced loading.

Altered temporomandibular joint function demonstrated conformational shape changes of the condylar head over time. Anterior displacement of the mandible (FWD), typically seen in orthodontic treatment of mandibular retrusion with bite jumping appliances [35], resulted in significant remodelling of the condylar head. Morphologically, there was an increased propensity for an irregular shaped condylar head with the loss of the typically ovoid or tear-drop appearance seen by Control animals (Figure 1). There was also a loss in the definition of the medial and lateral poles of the condylar head and the development of a noticeable notch on the posterior border of the condylar head (Figure 2). These changes were consistent with volumetric reduction noticed when examining the differences between total and posterior cartilage volumes of Control and FWD groups, with a significant reduction in FWD group (Table 1).

Posterior displacement of the mandible (BWD), typically seen in the treatment of mandibular prognathism with mandibular chin cup appliances [36], demonstrated differing results to the existing orthodontic literature [5,6] suggestive of significant condylar remodelling. Qualitatitively and quantitatively, minimal differences were noted between BWD and Control groups. This was consistent with previous studies that examined the

effects of differing loads on the mandibular cartilage which concluded an increased load on the cartilage. The condylar cartilage actually increased to adapt to the new functional requirement [7,8].

Though statistically there were no differences between BWD and FWD groups, trends were noticed graphically. Future investigations, with an increased sample size, will be required to demonstrate any significant differences.

This study has successfully demonstrated that condylar cartilage can be accurately assessed qualitatively and quantitatively. However the experimental method can be improved by providing animals a normal non-restrictive rodent diet, which is a harder consistency to imitate normal physiological function. The animals in this study did not exhibit the characteristic increase in weight as expected from pubertal animals, and even exhibited a significant loss in weights for the experimental animals. The application of a soft diet and the lack of pubertal growth, were confounding factors that could potentially influence cartilage volumes over time.

Additionally, efforts should be made to examine the feasibility of disarticulating the condyle to allow more efficient staining of the cartilage, and improve positioning of the sample in the MicroCT scanner. This may limit the need to use computer software to digitally reorientate the samples and remove irrelevant image distractions (adjacent musculoskeletal structures) prior to cartilage segmentation.

CONCLUSIONS:

This study demonstrated that the condylar cartilage of Sprague Dawley Rats could be successfully visualised and quantitatively measured made via enhanced MicroCT. The following conclusions may be made:

(6) Alterations in loads applied to the condylar head resulted in functional adaptations within the condylar head. This was represented as conformational changes in the shape of the condyle.

(7) Anterior displacement of the condylar head resulted in significant remodelling changes and reduction in cartilage and total volumes over time in comparison to normal and posterior displacement of the condyle.

(8) Differences between Anterior and Posterior displacement groups were not significant, however obvious trends were present and require further investigation.

(9) Though further refinements are required to improve this technique, this study has shown a new viable alternative to measuring condylar cartilage change.

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Attached Figures and Tables

Figure 50 - Qualitative Assessment of Condylar Heads - Superior View

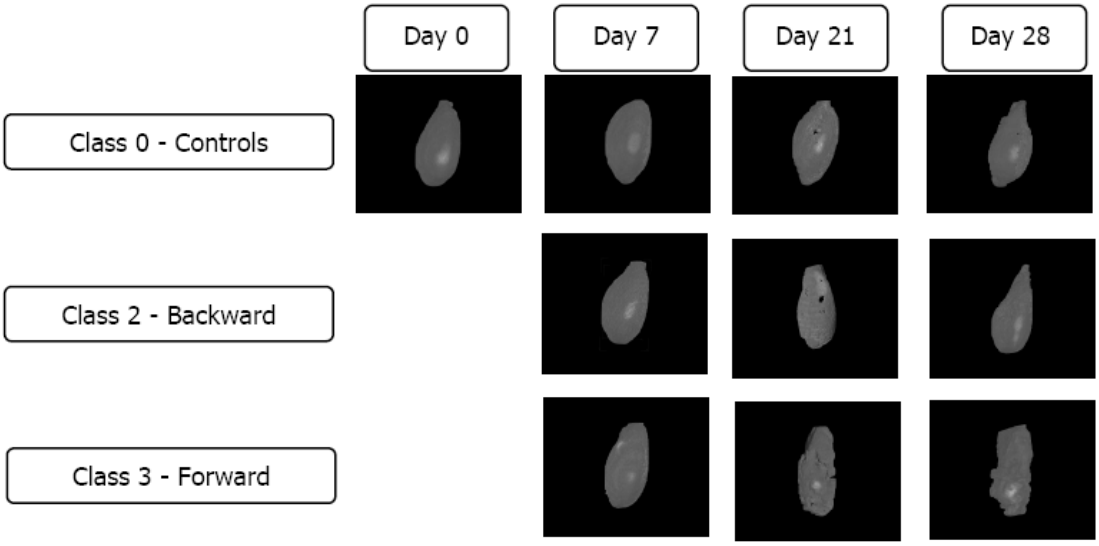
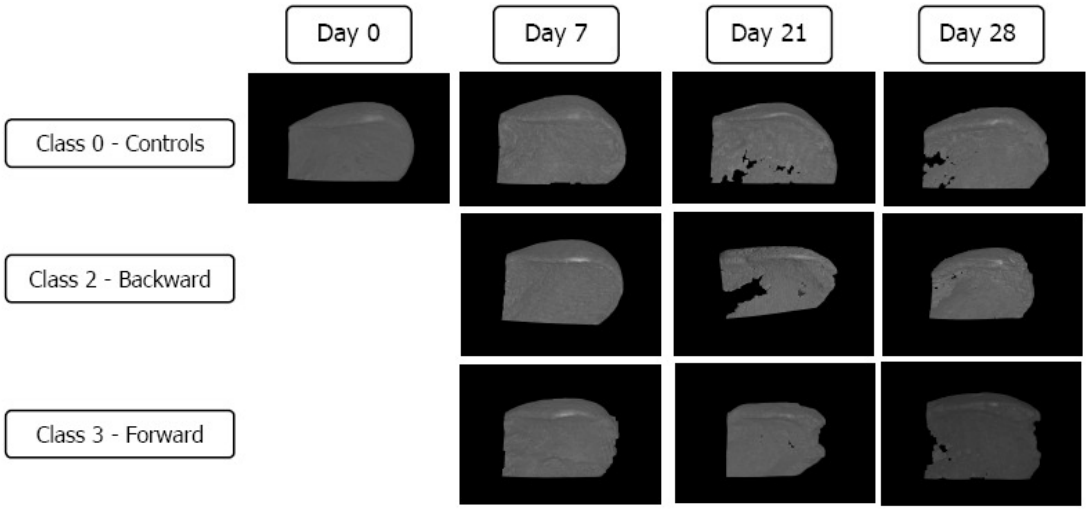


Figure 51 - Qualitative Assessment of Condylar Heads - Lateral View



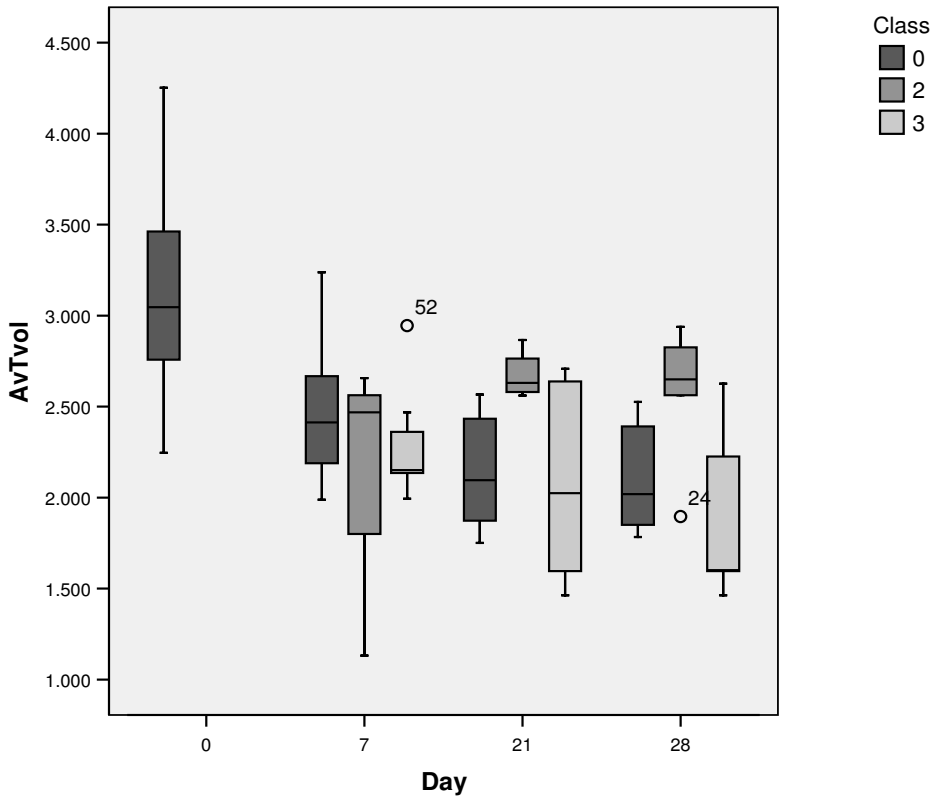


Figure 52 - Box plot graph of Total Volume data. Note the inverse trend between Class 2 and Class 3 over the Day 7-28 Period

Table 7- Univariate ANOVA of Volumetric changes

Dependent Variable (Average)	Day									Class						
	Estimated Marginal Means								P-value	Estimated Marginal Means						P-value
	0		7		21		28			0		2		3		
	Mean	SE	Mean	SE	Mean	SE	Mean	SE		Mean	SE	Mean	SE	Mean	SE	
Total Volume (AvTvol mm^3)	2.813[a]	0.092	2.417[a]	0.056	2.335[a]	0.053	2.218[a]	0.053	0.000	2.372	0.039	2.539	0.072	2.427	0.60	0.124
Total Cartilage Volume (AvTcart mm^3)	1.152[b]	0.053	0.940[b]	0.032	0.875[b]	0.031	0.927[b]	0.031	0.000	0.900[c]	0.022	0.980	0.042	1.041[c]	0.35	0.004
Posterior Total volume (avTvolP mm^3)	1.424[d]	0.087	1.262	0.053	1.134[d]	0.050	1.145[d]	0.050	0.014	1.195	0.036	1.340	0.068	1.190	0.056	0.136
Posterior Cartilage volume (AvPcart mm^3)	0.623[e]	0.055	0.495[e]	0.034	0.438[e]	0.032	0.498[e]	0.032	0.037	0.442[f]	0.023	0.540	0.043	0.559[f]	0.036	0.015

Bonferroni Adjustment for Multiple comparisons notes:

[a] – There is a difference between Day 0 and 7, 21, 28 in Total volumes only (p-value <0.002)
[b] – There is a difference between Day 0 and 7, 21, 28 in Total Cartilage volumes only (p-value <0.001)
[c] – There is a difference between Class 0 and 3 in Total Cartilage volumes only (p-value <0.004)
[d] – There is a difference between Day 0 and 21, 28 in Posterior Total volumes only (p-value <0.033)
[e] – There is a difference between Day 0 and 7, 21, 28 in Posterior Cartilage volumes only (p-value <0.001)
[f] – There is a difference between Class 0 and 3 in Posterior Cartilage volumes only (p-value <0.001)

Table 8 - Method Error Analysis

	Paired sample Correlations		Paired Differences				Error Analysis
Dependent Variable	**Correlation**	**P-Value**	**Mean**	**SD**	**SE**	**P-Value (2-tailed)**	**Co-efficient of variation**
Total Volume (Tvol)	0.951	0.000	0.008345	0.179576	0.022104	0.707	5.37%
Total Cartilage Volume (Tcart)	0.911	0.000	-0.017507	0.090388	0.011126	0.120	6.83%
Posterior Total Volume (TvolP)	0.934	0.000	0.008585	0.143247	0.017632	0.628	8.94%
Posterior Cartilage Volume (Pcart)	0.874	0.000	-0.019481	0.089769	0.011050	0.083	13.32%

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