Morphofunctional analysis of temporomandibular joint after bilateral discectomy and discopexy. Preclinical study

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Orientadores: Prof. Doutor Francisco João Salvado e Silva
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Tese especialmente elaborada para a obtenção do grau de Doutor em Medicina, Especialidade de Cirurgia Maxilofacial

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ÍNDICE

LISTA DE ABREVIATURAS .......................................................................................................................... v
RESUMO ...................................................................................................................................................... vii
ABSTRACT .................................................................................................................................................... xiii
LISTA DE PUBLICAÇÕES ........................................................................................................................... xix
LISTA DE COMUNICAÇÕES EM CONGRESSOS ................................................................................... xxi
CAPÍTULO 1. INTRODUÇÃO ..................................................................................................................... 1
CAPÍTULO 2. OBJETIVOS DO ESTUDO ................................................................................................... 11
CAPÍTULO 3. DEFINIR MODELO ANIMAL PARA ESTUDOS PRÉ-CLÍNICOS NA ÁREA DA ARTICULAÇÃO TEMPOROMANDIBULAR ......................................................................................... 13
CAPÍTULO 4. RECONSTRUÇÃO EM 3 DIMENSÕES DO DISCO DA ARTICULAÇÃO TEMPOROMANDIBULAR DA OVELHA BLACK MERINO .................................................................................. 33
CAPÍTULO 5. PROTOCOLO PARA MODELO DE INVESTIGAÇÃO PRÉ-CLÍNICA EM ARTICULAÇÃO TEMPOROMANDIBULAR RESPEITANDO AS ARRIVE GUIDELINES .......................................................................................................................... 37
CAPÍTULO 6. EFEITO DA DISCECTOMIA E DISCOPEXIA BILATERAL NA CINEMÁTICA MASTIGATÓRIA DA OVELHA BLACK MERINO. ESTUDO PRÉ-CLÍNICO RANDOMIZADO EM OCULTAÇÃO ...................................................................................................................... 55
CAPÍTULO 7. ANÁLISE HISTOLÓGICA, IMAGIOLÓGICA E DE MASSA CORPORAL APÓS DISCECTOMIA E DISCOPEXIA BILATERAL NUM ESTUDO PRÉ-CLÍNICO RANDOMIZADO EM OCULTAÇÃO .................................................................................. 77
CAPÍTULO 8. DISCUSSÃO GERAL E CONCLUSÕES ........................................................................ 103
  8.1. DISCUSSÃO GERAL .......................................................................................................................... 103
  8.2. IMPLICAÇÕES PARA FUTURA INVESTIGAÇÃO E CLÍNICA .......................................................... 108
  8.3. CONSIDERAÇÕES FINAIS .............................................................................................................. 109
AGRADECIMENTOS ................................................................................................................................. 111
PRÉMIOS E BOLSAS ................................................................................................................................. 117
REFERÊNCIAS ........................................................................................................................................... 119
FACSIMILE ................................................................................................................................................... 129
LISTA DE ABREVIATURAS

**Português**

3D – Tridimensional
ATM – Articulação Temporomandibular
DTM – Disfunção Temporomandibular
RM – Ressonância Magnética
RX – Raio X
TC – Tomografia Computorizada

**Inglês**

AC – Articular Cartilage
APT – Anteroposterior Tests
ARRIVE – Animal Research: Reporting of In Vivo Experiments
CT – Compression Tests CT
H&E – Hematoxylin and Eosin
MDT – Mediolateral Tests
OA – Osteoarthrosis
PCL – Polycaprolactone
PDS – Poly-p-Dioxanone
PDS – Poly- p-Dioxanone
PGS – Poly(Glycerol Sebacate)
PGs – Proteoglycans
RDC/TD – Research Diagnostic Criteria for Temporomandibular Disorders
SPIRIT – Standard Protocol Items: Recommendations for Interventional Trials
TEMPOJIMS – Temporomandibular Joint Interposal Material Study
THF – Tetrahydrofuran
TII – Temporomandibular Interpositional Implant TII
TMD – Temporomandibular Dysfunction
TMJ – Temporomandibular Joint
RESUMO

INTRODUÇÃO

A articulação temporomandibular (ATM) é a mais usada no corpo humano, realizando cerca de 2000 movimentos por dia, sendo essencial para as funções básicas do dia a dia como: mastigar, falar, sorrir ou bocejar. A ATM relaciona o côndilo mandibular com a cavidade glenoíde do osso temporal. Na interposição destas superfícies articulares encontra-se o disco intra-articular que permite absorver e distribuir as forças mastigatórias, diminuir as incongruências ósseas, contribuindo no movimento normal desta articulação. As disfunções da ATM (DTM) têm uma prevalência de cerca de 34%, e representam a principal causa de dor orofacial de origem não dentária, podendo estar associadas a morbidade elevada nos doentes com patologia mais avançada (categoria 3-5 na classificação de Dimitroulis). Nestes casos, o tratamento recomendado é predominantemente cirúrgico, sendo maioritariamente realizado através de discopexia ou discectomia. Os resultados destas intervenções constituem um atual tema de debate, principalmente no caso da discectomia, uma vez que a ATM fica sem o disco articular. Pela literatura científica existente, é possível verificar que não existe nenhum estudo randomizado, oculto, com grupo controlo, seja no homem ou no animal, que avalie o efeito destas intervenções na ATM.

OBJETIVOS

Assim, o objetivo principal desta dissertação foi avaliar o efeito da discectomia e discopexia na ATM em modelo animal (ovelha Black Merino), analisando o seu impacto histológico, radiológico, na mastigação e na cinemática ruminatória.

Objetivos específicos:

1. Definir o modelo animal apropriado para estudos pré-clínicos na área da ATM.
2. Caracterizar com rigor a anatomia e biomecânica do disco nativo da ovelha Black Merino.
3. Elaborar um desenho de estudo de investigação pré-clínico orientado à área da ATM, respeitando as ARRIVE guidelines.
4. Aplicar o modelo animal da ovelha Black Merino no desenho de estudo proposto.
5. Propor e testar variáveis piloto na área da cinemática ruminatória e tempo de mastigação para estudos pré-clínicos na ATM e avaliar o seu impacto após discectomia bilateral e discopexia bilateral na ovelha Black Merino.
6. Avaliar o impacto histológico e imagiológico da discectomia bilateral e discopexia bilateral na ovelha Black Merino.

MÉTODOS

Para alcançar os objetivos definidos, foram propostas e desenvolvidas as seguintes atividades:

1) Definição do modelo animal apropriado para estudos pré-clínicos na área da ATM: foram utilizadas 15 cabeças de ovelha Black Merino fêmeas, com 40 a 50 Kg, saudáveis. Na fase 1 foi efetuada uma descrição anatômica nos domínios cirúrgico, topográfico e histológico da ATM, comparando à articulação humana. Na fase 2 procedeu-se à análise histológica e biomecânica do disco articular após dissecção microcirúrgica dos discos. Para a análise histológica, as ATM foram removidas em bloco, descalcificadas em ácido fórmico, incluídas em parafina e coradas com hematoxilina & eosina e orceína. Para a avaliação biomecânica do disco articular, foram randomizados 9 discos, dos quais 3 testados à compressão, 3 à tensão anteroposterior e 3 à tensão mesiolateral.

2) Caracterizar com precisão a anatomia e biomecânica do disco nativo da ovelha Black Merino: foi realizada a remoção microcirúrgica de 6 discos articulares. Após submersão do disco numa solução impregnante, foi realizado um scanner do mesmo com um sistema de luz branca, reproduzindo-o com elevada precisão num modelo virtual em 3D.

3) Elaborar um desenho de estudo de investigação pré-clínico orientado à área da ATM, respeitando as ARRIVE guidelines: foi proposto um desenho de
estudo randomizado com 10 ovelhas e receptiva alocação em 3 intervenções: discectomia bilateral, discopexia bilateral e cirurgia placebo. Uma ovelha foi alocada a um grupo de reserva. Foi planeado um registo pré-operatório do peso, do tempo de mastigação de 150gr de ração e da cinemática ruminatória. Foi planeada também uma tomografia computorizada (TC) pré-cirúrgica. Os animais seriam intervencionados cirurgicamente e avaliados mensalmente, num total de 6 meses, sendo posteriormente sacrificados. Após sacrifício seria realizada nova TC, para avaliação imagiológica e preparada a avaliação histológica das articulações em ocultação para a intervenção.

4) Aplicar o modelo animal da ovelha Black Merino no desenho de estudo proposto: o estudo teve início em dezembro de 2015, o registo pré-operatório teve início em janeiro de 2016, as intervenções cirúrgicas foram realizadas durante o mês de fevereiro. Os animais foram acompanhados durante 6 meses com registos periódicos predefinidos. Os animais foram sacrificados em agosto de 2016.

5) Testar variáveis piloto na área da mastigação e da cinemática ruminatória para estudos pré-clínicos na ATM: diariamente, às 08:30h, os animais foram colocados em divisória própria com abertura frontal, previamente desenvolvida para avaliação do tempo de mastigação e da cinemática ruminatória em ovelha. Seguiu-se uma cronometragem do tempo para comer uma dose controlada de 150gr de ração Rico Gado A3®, registando o respetivo tempo de mastigação. Os animais foram colocados no campo, ao ar livre, e às 13:00h voltaram a ser instalados nas divisórias para serem filmados 15 ciclos ruminatórios. Com recurso a software apropriado, foi efetuado um mapa do trajeto da mandíbula durante a ruminação e obtido o tempo de cada ciclo ruminatório, a forma geométrica e a área de ruminação. Após este registo os animais foram pesados numa balança devidamente calibrada.

6) Avaliar o impacto histológico e imagiológico da discectomia bilateral e discopexia bilateral na ovelha Black Merino: após o sacrifício das ovelhas, foi realizada TC ao crânio, separados os blocos de cada ATM, e preparados para
análise histológica. Em ocultação, os diferentes avaliadores pontuaram, respetivamente, as articulações com escalas apropriadas.

RESULTADOS

1) O acesso cirúrgico à ATM da ovelha *Black Merino* realiza-se através de uma incisão pré-auricular, dissecção por planos até à cápsula sinovial. As dimensões das estruturas anatômicas envolvidas (*e.g.* fossa temporal, côndilo mandibular, espaço interarticular e disco articular) são aproximadas às do humano, tal como a histologia e a biomecânica do disco que apresentam idênticas semelhanças. No entanto, registou-se um perfil ligeiramente côncavo do côndilo mandibular da ovelha, que na raça humana é convexo.

2) Foi possível, através do método anteriormente descrito, replicar com rigor a morfologia do disco nativo da ovelha *Black Merino*. O disco apresentou em média 20.93±1.33mm no sentido mesiolateral e 11.2±0.78mm no sentido anteroposterior. A média da espessura da banda anterior foi 1.03±0.06mm, a zona central 0.76±0.08mm e a banda posterior 1.23±0.07mm. A morfologia em fresco evidenciou uma ligeira convexidade da superfície inferior, correspondendo a um perfil adaptativo ao côndilo mandibular.

3) O desenho do estudo randomizado, oculto com alocação em 3 intervenções bilaterais, respeitando as ARRIVE *guidelines*, foi executado com rigor e provou ser exequível na ovelha *Black Merino*. A avaliação do tempo para mastigar uma dose fixa de 150gr de ração mostrou ser adequada para avaliar o tempo de mastigação inicial. A captação vídeo de 15 ciclos ruminatórios foi reprodutível, tendo o animal demonstrado capacidade de se manter estático no processo de ruminação, nas divisões desenvolvidas para este efeito.

4) Foram detetadas diversas alterações histológicas nos grupos da discectomia e da discopexia. O grupo controlo apresentou as características da ATM normal previamente descrita. No grupo da discectomia verificou-se fibrilhação e perda da
camada superficial laminar, com aumento marcado da população de proteoglicanos e invasão vascular da zona intermédia. Em alguns casos observou-se remodelação subcondral. A avaliação em ocultação mostrou diferenças estatisticamente significativas entre este grupo e o grupo de controlo. Na discopexia verificou-se ligeira fibrilhação sem perda da continuidade da superfície laminar, havendo aumento do número de proteoglicanos e da densidade celular. Na avaliação em ocultação não se encontraram diferenças estatisticamente significativas entre o grupo da discopexia e o de controlo.

5) A avaliação em ocultação das imagens de TC mostrou diferenças estatisticamente significativas apenas para a discectomia ($R^2$ correspondendo a 92,9% de degeneração na avaliação global). Na discopexia foram encontradas ligeiras diferenças sem significado estatístico.

6) Em ambas as técnicas cirúrgicas houve alterações estatisticamente significativas do tempo de mastigação, no primeiro mês após a cirurgia, que foram normalizando e, após 2 meses, deixaram de ser significativas. Na avaliação da ruminação no grupo da discopexia, foi encontrada uma diferença estatisticamente significativa, com aumento do ciclo de ruminação no 5º mês pós cirurgia. Não se encontraram outras alterações significativas, ao longo dos meses, na avaliação do tempo de cada ciclo de ruminação e respetiva área.

7) Após a intervenção cirúrgica, os animais do grupo da discectomia e discopexia perderam peso até ao final do 1º mês. No decorrer do estudo, o grupo da discectomia apenas conseguiu recuperar o peso inicial, enquanto o grupo da discopexia continuou em progressão de peso, acompanhando o perfil do grupo de controlo. No presente estudo, não houve diferenças estatisticamente significativas no peso dos animais dos diferentes grupos estudados.
CONCLUSÕES

A ovelha *Black Merino* é um animal com características apropriadas para ser utilizado em estudos pré-clínicos da ATM, por apresentar uma anatomia cirúrgica e histológica semelhante ao humano, e o disco apresentar morfologia e comportamento biomecânico idêntico. Por ser um animal ruminante, com consequente aumento do número de movimentos da ATM, poderá contribuir favoravelmente em estudos posteriores para testar potenciais biomateriais ou próteses da ATM (*e.g.* testar fadiga do material). O desenho de estudo proposto randomizado, oculto, com intervenções cirúrgicas bilaterais verificou-se exequível e reprodutível, para investigação na área da ATM em ovelha *Black Merino*. A discectomia promoveu um processo degenerativo severo, na análise histológica e radiológica, com uma repercussão significativa no tempo inicial de mastigação apenas no 1º mês pós cirurgia. A discopexia não induziu um dano degenerativo significativo na articulação. A bioengenharia de tecidos e a medicina regenerativa poderão desempenhar um papel significativo na regeneração do disco articular, contribuindo para uma melhoria das técnicas cirúrgicas atuais, nomeadamente a discectomia.
ABSTRACT

INTRODUCTION

The temporomandibular joint (TMJ) is the most used joint in the human body, with over 2000 movements per day, being essential for everyday functions (e.g. mastication, speech, deglutition and yawning). The TMJ is responsible for the relation between the mandibular condyle and glenoid fossa of temporal bone. This joint contains an articular disc, an important functional unit interposed between the bony structures, contributing for a congruent movement of this joint. TMJ disorders (TMD) have a prevalence of 34% and represent the main non-tooth origin cause of orofacial pain, leading to high morbidity in severe cases (category 3-5 Dimitroulis classification). In those cases, the treatment is mostly surgical, being the common surgical options discopexy or discectomy. The outcomes of these interventions are a topic of debate, namely in discectomy, once TMJ is left without the TMJ disc. Moreover, in the available scientific literature it is not possible to state any randomized study, blind and with control group, both in human or animals, evaluating the effect of those interventions in TMJ.

OBJECTIVES

The main goal of this dissertation was evaluating the effect of TMJ discectomy and discopexy in animal model (Black Merino sheep), examining their impact in histologic, imaging and kinematic outcomes.

Specific goals:

1. Characterisation of the adequate animal model for preclinical studies in the TMJ.
2. Characterize the anatomy and biomechanics of the native TMJ disc of Black Merino sheep.
3. Develop a study design for preclinical investigation in the TMJ, according to the ARRIVE guidelines.
4. Application of the proposed study design on the *Black Merino* animal model.

5. Propose and test pilot outcomes on the kinematic mastication for preclinical studies in the TMJ and evaluating their impact on bilateral discectomy and bilateral discopexy in *Black Merino* sheep.

6. Evaluating the histologic and imaging impact of bilateral discectomy and bilateral discopexy in *Black Merino* sheep.

**METHODS**

To achieve the define goals, the following activities were developed and proposed:

1) **Characterisation of the adequate animal model for preclinical studies in the TMJ:** 15 female *Black Merino* sheep heads of healthy animals with 40-50 Kg were used to describe the surgical, topographic and histologic anatomy of the TMJ, comparing with the human joint. In a second phase, histologic and biomechanical analysis of the disc were performed. For the anatomical characterisation, a surgical dissection was performed exposing and identifying the TMJ structures. To analyse the TMJ disc, a microsurgical dissection was performed and the TMJ discs were submersed in an impregnated solution (Colorbond), allowing a 3D scanning preserving the morphology of the native disc. To the histologic analysis, the TMJ was removed in block, decalcified in formic acid, included in paraffin and stained with hematoxilin & eosin and orcein. For the biomechanical evaluation, 9 discs were randomized (3 were tested to compression, 3 to anteroposterior strain and 3 to mesiolateral strain).

2) **Characterize the anatomy and biomechanics of the native TMJ disc of *Black Merino* sheep:** a microsurgical extraction of 6 discs was completed, removing all the muscular attachments. The discs were submersed in an impregnate solution to preserve their native morphology. A white light system scanner was used, replicating a 3D virtual model with high precision.

3) **Develop a study design for preclinical investigation in the TMJ, according to the ARRIVE guidelines:** a randomized preclinical trial with 10 sheep was
proposed with allocation in 3 intervention groups: bilateral discectomy, bilateral discopexy, sham surgery and one reserve sheep was allocated in a reserve group. A baseline pre-intervention record of body mass, mastication time for 150gr of pellets and rumination kinetics was included. In addition, imaging by computed tomography (CT) was suggested before surgical intervention. It was proposed that the animals would be submitted to surgical intervention and evaluated every 6 months, being posteriorly sacrificed. After sacrifice, a new CT should be done for imaging scoring and discs ought to be prepared for the histologic evaluation.

4) Application of the proposed study design on the Black Merino animal model: the study started in December 2015, the baseline records occurred in January 2016, and the surgical interventions took place in February 2016. According to the established protocol, the animals were monitored for 6 months (with various assessments being performed in specific time points) and were sacrificed in August 2016.

5) Propose and test pilot outcomes on the kinematic mastication for preclinical studies in the TMJ and evaluating their impact on bilateral discectomy and bilateral discopexy in Black Merino sheep: nine specific cages were constructed with a frontal window where all the animals were placed at 08:30 am. The time to eat 150gr of dry pellets Rico Gado A3® was assessed by a chronometer. Following, the animals were placed in their natural habitat and around 01:00 pm they returned to the boxes to record the kinetic rumination of 15 cycles. With adequate software, the trajectory of the jaw was designed during rumination and the time for each cycle was calculated, as well as the ruminant area. Next, the body mass was obtained in a certified balance.

6) Evaluating the histologic and imaging impact of bilateral discectomy and bilateral discopexy in Black Merino sheep: after sacrifice, a cranium CT was performed and the TMJ block was removed. Each block was prepared for histology. In occultation, the different evaluators scored the joint using appropriate scales.
RESULTS

1) The obtained surgical anatomy was similar to the human, with a direct access to TMJ through a pre-auricular incision. The size of the anatomic structures (e.g. temporal fossa, mandibular condyle, inter-articular space and TMJ disc) were also similar to the human. In addition, high similarity was also obtained in the disc histology and biomechanics. A slight concave mandibular condyle was noticed, which is convex in humans.

2) With the proposed method, it was possible to accurately replicate the morphology of the native TMJ disc of *Black Merino* sheep. The average size was 20.93±1.33mm in the mesiolateral orientation and 11.2±0.78mm in anteroposterior orientation. The thickness of the anterior band was 1.03±0.06mm, the central zone 0.76±0.08mm and the posterior band 1.23±0.07mm. The morphology of the fresh disc showed a slight convexity of the inferior surface, consistent to an adaptive outline to the mandibular condyle.

3) The randomized and blinded study design, with allocation in 3 different bilateral interventions, respecting the ARRIVE guidelines was executed with accuracy and proved to be feasible in *Black Merino* sheep. The video caption of 15 rumination cycles was achievable, showing this animal the ability to stand still during the rumination.

4) Various histologic changes were noticed in the discectomy and discopexy groups. As expected, the control group presented the main characteristics of the normal TMJ. In the discectomy group, fibrillation and loss of typical laminar structure, with an increased population of proteoglycan stain in all layers and vascular invasion, were observed. In some cases, osteochondral changes were also noticed. The blinded scoring showed statistical significance for the discectomy group only. While no statistical significance, with no loss of the laminar surface, was detected in the discopexy group, a marked proteoglycan increase was noticed.
5) The blinded evaluation of CT images showed statistical significance only for the discectomy group \((R^2\) corresponding to 92.9\% of degeneration for global appreciation). In the discopexy group, slight differences were noticed without reaching statistical significance.

6) For both surgical techniques, significant changes were noticed for mastication time in the first month post-operative. Those changes gradually returned to normal and 6 months after surgery no changes were observed. No rumination movements were detected in T1 and T2 after surgery in the discectomy group. In rumination evaluation, a significant difference was stated in T5 for discopexy group with an increased time \(\text{per cycle}\). No other changes occurred, neither for rumination time or area.

7) In the first month after surgery, discectomy and discopexy groups loss body mass. However, with the progress of the study, those animals were capable to increase body mass; discectomy group was only capable to return to original weight, and discopexy groups could follow the control group increasing 8\% and 8.2\% respectively. No significant changes were noticed in the different groups.

CONCLUSIONS

The \textit{Black Merino} sheep has proven to be an adequate animal model to conduct TMJ preclinical trials, considering the surgical anatomy, biomechanics and histology like human. Because it is a ruminant animal, it may have an increased value in further studies, examining interpositional biomaterials or TMJ prosthesis, and allowing to accurately test the material stress. The proposed randomized, blinded, with bilateral interventions study was feasible and reproducible for TMJ investigation in Black Merino sheep. Discectomy promoted a severe degenerative process in histologic and radiologic analysis, with a repercussion in mastication (only in the first month post-surgery). Discopexy did not induce significant degenerative changes in the TMJ. With a significant role in tissue regeneration, bioengineering and regenerative medicine will be critical to increase and optimize the current surgical techniques, mostly discectomy.
LISTA DE PUBLICAÇÕES

Durante esta investigação foram publicados diversos artigos científicos tendo como objetivo partilhar, com a comunidade científica, o conhecimento obtido ao longo deste estudo.

• Ângelo, David F; Morouço, Pedro; Alves, Nuno; Viana, Tânia; Santos, Fábio; González, Raúl; Monje, Florencio; Macias, Domingos; Carrapiço, Belmira; Sousa, Rita; Gonçalves, Sandra; Salvado, Francisco. Animal model for temporomandibular joint research: morphological, histological and biomechanical characterization of the Black Merino sheep joint disc. Morphologie. 2016 Dec;100(331):223-233.

• Ângelo, David F; Morouço, Pedro; Alves, Nuno; Carvalho, Tânia; Bonaparte Dolores; Carrapiço, Belmira; Monje, Florencio; Furtado, Ivo; Salvado, Francisco. Novel approach for 3D virtual model of TMJ disc morphology. Preliminary results. Surg Radiol Anat. 2016 Jan;38(1):5-47.

• Ângelo, David F; Monje, Florencio; González, Raúl; Little, Christopher B; Mónico, Lisete; Pinho, Mário; Santos, Fábio; Carrapiço, Belmira; Cavaco, Sandra; Morouço, Pedro; Alves, Nuno; Moura, Carla; Wang, Yadong; Jeffries, Eric; Gao, Jin; Sousa, Rita; Lucas, Lia; Caldeira, Daniel; Salvado, Francisco. Bioengineered Temporomandibular Joint Disk Implants: Study Protocol for a Two-Phase Exploratory Randomized Preclinical Pilot Trial in 18 Black Merino Sheep (TEMPOJIMS). JMIR Res Protoc. 2017 Mar 2;6(3):e37.

• Ângelo, David F; Monje Florencio; González, Raúl; Mónico, Lisete; Moura, Carla; Francisco, Luís; Sanz, David; Alves, Nuno; Salvado, Francisco; Morouço, Pedro. Effects of bilateral discectomy and bilateral discopexy on Black Merino Sheep rumination kinematics: TEMPOJIMS – a randomized preclinical trial. Accepted in Journal of Cranio and Maxillofacial Surgery.

• Ângelo, David F; Monje Florencio; González-Garcia Raúl, Morouço Pedro; Sousa Rita; Neto Lia; Caldeira Inês; M. Smith Margaret; M. Smith Susan; Sanz David; Carvalho Fábio; Carrapiço Belmira; Cavaco Sandra, Pinho Mário; Wang Yadong; Jeffries Eric; Gao Jin; Moura Carla; Alves Nuno; Mónico Lisete; Salvado Francisco; Little Christopher B. A Pilot Preclinical Randomized Controlled Trial
of Bilateral Discectomy versus Bilateral Discopexy in Black Merino Sheep Temporomandibular Joint: TEMPOJIMS Phase 1 - Histologic, Imaging and Body Weight results. Accepted in JCMFS.
LISTA DE COMUNICAÇÕES EM CONGRESSOS

Durante a investigação foi possível obter diversos resultados preliminares, que permitiram ir divulgando os resultados em congressos, assim como discutir várias ideias com conferencistas, contribuindo para a melhoria contínua do projeto.

- Ângelo, David F, *et al.* Temporomandibular joint interposial material study - TEMPOJIMS. 24 Congreso nacional de cirurgia oral y maxilofacial. Málaga - Espanha. 10 de junho de 2017
- Ângelo, David F, *et al.* Bioengenieria de tejidos en articulacion temporomandibular. 4º curso de regeneración y reconstruction óssea de los maxilares - Sociedade espanola de cirurgia oral y maxilofacial. Madrid - Espanha. 11 de novembro de 2016

CAPÍTULO 1. INTRODUÇÃO

A articulação temporomandibular (ATM) é responsável pela relação do côndilo mandibular com a cavidade glenoíde do osso temporal [1]. É uma articulação sinovial classificada como bicondilo-meniscartrose, sendo a única articulação do corpo humano que conjuga movimentos de rotação e translação (Figura 1) [2].

Figura 1. (A) Vista lateral da ATM. (B) Movimento de abertura da boca com detalhe para a posição do disco articular [3].

À par de outras articulações móveis, a ATM é caracterizada por ter uma membrana sinovial que reveste a cápsula articular e engloba as superfícies articulares. A articulação é dividida em compartimento superior e inferior pelo disco articular fibrocartilaginoso, permitindo um deslizamento suave destas estruturas ósseas. O disco articular é composto por uma população mista de fibroblastos e condrócitos que produzem maioritariamente colágeno tipo 1 [4]. O disco é, na sua grande maioria, avascular, aneural e alinfático [4]. As características da ATM e do disco articular são únicas e não devem ser comparadas, por exemplo, ao menisco do joelho [5]. O disco articular tem, como principais funções: lubrificar a ATM, absorver/distribuir as forças mastigatórias e estabilizar o movimento desta articulação [6]. Em média, no adulto, o disco tem 19mm no sentido mesiolateral e 13mm no sentido anteroposterior [6]. A superfície inferior do disco apresenta uma excelente adaptação à forma do côndilo articular, e reduz assim os níveis de atrito nos movimentos de rotação e translação [7]. A membrana sinovial desempenha um papel importante na função da articulação, nomeadamente na produção de líquido sinovial, principal fonte de nutrientes para o disco e cartilagem articular. A
cartilagem que reveste a superfície óssea é não hialina, tornando esta articulação atípica [8]. Os condrócitos que caracterizam a cartilagem hialina podem ser identificados em 4 zonas na cartilagem: superficial, média, profunda e calcificada. Em contraste, a cartilagem da ATM tem zonas de fibrocartilagem e de cartilagem tipo hialina, representadas por uma zona fibrosa superficial fibroproliferativa e uma zona inferior com características hialinas. Esta zona inferior apresenta sobretudo colagénio tipo 1 e fibroblastos. Na camada inferior são encontradas células mesenquimais indiferenciadas, que constituem uma zona proliferativa de reserva para a camada fibrosa superficial [8]. Esta articulação desempenha uma função importante no crescimento da mandíbula, pois o côndilo tem uma área de atividade de crescimento [1]. No recém-nascido, a fossa mandibular é aplanada e não existe eminência articular. Só após a erupção dos primeiros dentes definitivos, por volta dos 6-7 anos de idade, a eminência articular começa a ser mais proeminente e a fossa temporal a ser moldada pelo côndilo [9]. A ATM é a articulação mais utilizada do corpo humano, contabilizando cerca de 2000 movimentos por dia e é essencial para manter as funções básicas oromaxilares do dia a dia (e.g. mastigar, falar, deglutir e bocejar), e está dependente do movimento indissociável das duas articulações (direita e esquerda), particularidade exclusiva da ATM [10].

As disfunções temporomandibulares (DTM) podem resultar de diversos fatores etiológicos: trauma [11], perda de dentes [12], parafunções [13], infeção [14], autoimunidade [15], sobrecarga articular [16] e diminuição da respetiva lubrificação [17]. As DTM representam a principal causa de dor orofacial de origem não dentária no humano [18,19]. Estas disfunções têm assumido uma importância crescente na comunidade médica, pela relevante prevalência na população (5-32,5%) [19–29], pelo grande impacto na qualidade de vida dos doentes [30–32] e custos importantes para os sistemas de saúde [33]. Prevê-se que no ano de 2030 dupliquem as intervenções cirúrgicas com próteses totais da ATM nos Estados Unidos [34]. Concomitantemente, a comunidade científica tem-se debruçado exaustivamente sobre este tema, contribuindo para um aumento evidente do número de trabalhos publicados na Web of Science® (Figura 2).
Capítulo 1. Introdução

Existem diversas classificações para as DTM. A classificação Research Diagnostic Criteria for Temporomandibular Disorders (RDC/TMD), publicada em 1992, continua a ser a mais aceite. Avalia os doentes em dois eixos [35]: o eixo 1 está orientado para a patologia funcional e o eixo 2 consiste numa avaliação psicossocial do doente. A avaliação RDC/TMD foi elaborada principalmente para doentes não cirúrgicos e, embora a sua utilização na prática clínica seja reduzida, ela mantém um papel importante na área da investigação [36]. Na tabela 1 é apresentado o eixo 1 da RDC/TMD. O eixo 2, por ser orientado para a avaliação psicossocial, não foi apresentado. Esta classificação foi revista em 2014 [37], mas não se encontra ainda validada para a língua portuguesa pelo que se consideramos a versão de 1992.

<table>
<thead>
<tr>
<th>Grupo RDC/TMD</th>
<th>Diagnóstico</th>
<th>Definição</th>
<th>Critério diagnóstico</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Ia</td>
<td>Dor miofascial</td>
<td>1. Dor mandibular, têmporas, face, área pré auricular ou ouvido, em repouso ou função; mais</td>
</tr>
<tr>
<td></td>
<td>Ib</td>
<td>Dor miofascial com limitação da abertura da boca</td>
<td>2. Dor após palpação em 3 dos seguintes 20 locais (lado direito e esquerdo contam como áreas separadas): músculo temporal zona anterior, músculo temporal zona média, músculo temporal zona posterior, corpo do masséter, inserção do masséter, região posterior da mandíbula, região submandibular, região do pterigoideu lateral, tendão do músculo temporal.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dor de origem muscular, incluindo zonas de contratura muscular.</td>
<td>1. Dor miofascial como definida em Ia; mais</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dor de origem muscular, incluindo zonas de contratura muscular e limitação da abertura da boca</td>
<td>2. Abertura oral espontânea, sem dor inferior a 40mm; mais</td>
</tr>
</tbody>
</table>
Capítulo 1. Introdução

|   | IIA | Deslocamento do disco com redução | O disco está deslocado da sua posição entre o cóndilo e a eminência. O deslocamento pode ser anterior, posterior, medial ou lateral, mas reduz em abertura total, normalmente havendo um estalido. Se este diagnóstico for acompanhado de dor na articulação, o diagnóstico será de artralgia (IIla) ou de osteoartrite (IIlb). | O disco está deslocado da sua posição entre o cóndilo e a eminência, associado a limitação da abertura da boca. | 1. (a) estalido recíproco que deve ocorrer pelo menos após 5mm da abertura máxima e no movimento de fechar a boca. Este estalido deve ser eliminado no movimento de protusão. Este processo deve ser reprodutível em pelo menos 3 aberturas; ou (b) estalido na abertura ou fecho da boca, em pelo menos 2 aberturas consecutivas, ou estalido em movimento lateral e protusão reprodutível, em pelo menos 2 aberturas. |
|   | IIb | Deslocamento do disco sem redução com limitação da abertura da boca | O disco está deslocado da sua posição entre o cóndilo e a eminência, sem associação a limitação da abertura da boca. | 1. História de limitação progressiva da abertura da boca; mais 2. Abertura espontânea < 35mm; mais 3. Abertura oral forçada melhora < 4mm; mais 4. movimento lateral < 7mm; mais 5. Abertura com desvio sem correção ipsilateral; mais 6. (a) sem presença de estalidos ou (b) estalidos que não se incluam nos critérios de IIa. |
|   | IIc | Deslocamento do disco sem redução e sem limitação da abertura da boca | O disco está deslocado da sua posição entre o cóndilo e a eminência. | 1. História de limitação progressiva da abertura da boca; mais 2. Abertura espontânea > 35mm; mais 3. Abertura oral forçada melhora > 5mm; mais 4. movimento lateral >7mm; mais 5. (a) presença de estalidos que não se incluam nos critérios de IIa. |

| III | IIIa | Artralgia | Dor ou tumefação da cápsula sinovial da ATM | 1. Dor à palpação do polo lateral ou zona retrodiscal; mais 2. Uma ou mais de seguintes situações: (a) dor na zona da ATM, (b) dor articular na abertura espontânea, (c) dor na abertura forçada, (d) dor no movimentos laterais. 3. Para artralgia simples não pode haver crepitação. |
| IIIb | Osteoartrite da ATM | Inflamação articular que resulta em processo degenerativo das estruturas articulares | 1. Artralgia; mais 2. Crepitação. |
| IIIc | Osteoartrose da ATM | Alteração degenerativa onde a morfologia articular está alterada | 1. Inexistência dos sintomas de artralgia, inexistência de dor articular ou de dor na abertura espontânea, ou nos movimentos de lateralidade. 2. Crepitação |

Tabela 1. Eixo I da classificação RDC/TMD [35].
Em 1989, Wilkes et al propôs outra classificação (Tabela 2), que se veio a tornar amplamente aceite para a classificação das DTM intra-articulares [38]. O sistema de Wilkes et al engloba uma avaliação clínica, radiológica e cirúrgica. No entanto, esta classificação está limitada aos processos de osteoartrite e aos deslocamentos do disco articular, não sendo mencionados processos mais severos (e.g. anquiloses da ATM, tumores da ATM) ou as DTM ligeiras com envolvimento muscular. Para estes casos é necessário recorrer a outras subclassificações [39,40] que, por não serem objetos de estudo do presente trabalho, não serão abordadas nesta dissertação.

<table>
<thead>
<tr>
<th>Estágio</th>
<th>Sinais e sintomas</th>
<th>Achados imagiológicos</th>
<th>Achados cirúrgicos</th>
</tr>
</thead>
</table>

**Tabela 2.** Classificação de Wilkes et al [38].

Mais recentemente, Dimitroulis publicou uma classificação simples, atualizada e abrangente das disfunções temporomandibulares [36], que se encontra dividida em cinco categorias (Tabela 3). Esta classificação engloba os diferentes tipos de DTM de um modo organizado, expondo as diversas propostas terapêuticas em relação à categoria. Assim, na prossecução dos objetivos da presente tese, foi adotada esta classificação como referência.
Capítulo 1. Introdução

<table>
<thead>
<tr>
<th>Categoria 1</th>
<th>ATM Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Não é necessário procedimento cirúrgico.</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Categoria 2</th>
<th>ATM com danos minor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Todos os componentes estruturais estão preservados.</td>
<td></td>
</tr>
<tr>
<td>Indicação para lavagem articular com artrocentese / artroscopia</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Categoria 3</th>
<th>ATM com danos moderados</th>
</tr>
</thead>
<tbody>
<tr>
<td>A maioria dos componentes estruturais está preservada.</td>
<td></td>
</tr>
<tr>
<td>Indicação para artroscopia cirúrgica / artroplastia ou discectomia.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Categoria 4</th>
<th>ATM com danos severos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poucos componentes estruturais estão preservados.</td>
<td></td>
</tr>
<tr>
<td>Indicação para artroplastia, discsectomia ou condilectomia.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Categoria 5</th>
<th>ATM com danos catastróficos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sem componentes preservados.</td>
<td></td>
</tr>
<tr>
<td>Indicação para ressecção da ATM ou substituição por prótese total.</td>
<td></td>
</tr>
</tbody>
</table>

Tabela 3. Classificação das disfunções da ATM por Dimitroulis [36].

mastigação. A abertura oral está normalmente diminuída por receio de abrir e deslocar a mandíbula ou devido à dor intensa. Na abertura oral verifica-se um desvio para o lado afetado, o que poderá significar um deslocamento anterior do disco sem redução. Pela limitação da abertura da boca, normalmente, não existem estalidos. Os exames complementares de imagem podem não evidenciar alterações. Na RM há normalmente deslocamento do disco sem redução, associada ou não, a deformação do disco e/ou hipertrofia da eminência articular. Estes doentes podem beneficiar de artroscopia cirúrgica, condilectomia funcional, artroplastia da ATM com reposicionamento do disco, discectomia, eminectomia ou redução aberta de fratura do côndilo com osteosíntese rígida.

Na categoria 4, existem alterações severas da ATM onde poucas estruturas articulares podem ser preservadas. Nesta categoria, os doentes apresentam dor constante com crepitação dolorosa da ATM e limitação moderada da abertura da boca. Mastigar é difícil e bocejar desencadeia frequentemente dor intensa. Os exames complementares de imagem mostram sinais de alterações morfológicas do côndilo, tais como aplanamento ou afilamento. Estas alterações são melhor visualizadas na TC. A RM mostra danos degenerativos severos, o disco deformado e/ou deslocado, podendo haver em vários casos perfuração. Alterações degenerativas da ATM como a presença de osteófitos, de pequenos quistos subcondrais com perda ou diminuição da fibrocartilagem podem ser observados na TC. O doente apresenta normalmente um quadro típico de disfunção intra-articular com osteoartrite recente. Nesta categoria podem estar incluídos casos de doenças metabólicas, auto-imunes ou inflamatórias da ATM. As opções terapêuticas recomendadas são discopexia, discectomia da ATM com ou sem desbridamento do espaço intra-articular, ou condilectomia funcional. Na categoria 5 estão os casos com alterações muito graves da ATM. Estes doentes apresentam dor incapacitante, crepitação e incapacidade de mastigar sólidos. Os exames complementares imagiológicos evidenciam alterações degenerativas óbvias, com superfície irregular e quistos subcondrais. A RM mostra alterações severas do disco, que, por vezes, não se visualizam corretamente pelo sinal hipointenso do côndilo deformado. Estes doentes apresentam osteoartrite ou patologia degenerativa severa que pode estar associada a cirurgias prévias à ATM. Quando não existe dor, ou quando esta é tolerável, o doente pode ter osteoartrose, anquilose óssea ou patologia tumoral na
Capítulo 1. Introdução

ATM. Estes doentes podem beneficiar de discectomia, condilectomia ou prótese total da ATM.

Tendo como referência a referida classificação, o principal objetivo desta investigação consistiu em alcançar um progresso clínico e científico na compreensão do efeito da discectomia e discopexia na ATM.

Historicamente, a discectomia da ATM (Figura 3) é a técnica cirúrgica mais realizada para tratamento de patologia intra-articular [41].

Figura 3. Doente na categoria 4 da classificação de Dimitroulis com fragmentação do segmento lateral do disco. O tratamento cirúrgico proposto foi discectomia da ATM esquerda. (A) acesso à ATM via pré auricular; (B) ATM após ser removido o disco (discectomia) e (C) elemento principal do disco. Não foi fotografado o fragmento lateral do disco.

Com vários estudos a evidenciar resultados satisfatórios pós-discectomia [42–46], esta técnica continua a ser utilizada com frequência, sendo, no entanto, controversa por eliminar da articulação um elemento estrutural importante, o disco articular. Vários grupos tentaram, através de estudos pré-clínicos [47–52], compreender o efeito histológico da discectomia, mas os resultados foram disparens, tornando-os inconclusivos. A heterogeneidade dos resultados pode estar associada a: (1) uso de diferentes modelos de animais [53–56], (2) desenhos de estudo pouco rigorosos [48,56,57], (3) uso da articulação contralateral como controlo [48,56], (4) limitação das variáveis estudadas à radiologia e histologia [48,54,56]. Assim, decorrente das referidas limitações, foi objetivo inicial do presente trabalho definir: o modelo animal ideal para conduzir investigações na área da ATM, desenhar um estudo pré-clínico randomizado, oculto, com grupo controlo sham e, respeitando as ARRIVE
Capítulo 1. Introdução

guidelines [58] que possa contribuir como modelo para futuras investigações nesta área, e estudar o efeito da discectomia e discopexia com intervenções bilaterais.

Os estudos pré-clínicos assumem um papel importante para o desenvolvimento da medicina, representando uma indispensável fronteira entre a investigação in vitro e os ensaios clínicos em humanos. Um fármaco ou dispositivo médico inicia o seu desenvolvimento com múltiplos testes e ensaios in vitro. Após verificados os perfis de toxicidade e/ou viabilidade celular, seguem-se estudos em animais para avaliar o comportamento do fármaco ou dispositivo (e.g. metabólico, farmacocinético, farmacodinâmico e perfis de segurança). Normalmente, estes testes são realizados em dois tipos diferentes de animais: roedores e não-roedores, existindo vários tipos de modelo animal para realizar investigação pré-clínica. No entanto, recorrendo ao princípio de Krogh [59], existem animais que são mais indicados no estudo de determinado tipo de problemas, tornando muitas vezes a sua escolha complexa. A reprodução de patologia articular em animais deve ter em consideração a anatomia, biomecânica e comportamento animal para reduzir possíveis erros nos estudos. Assim, a escolha do animal revela-se determinante para o desenvolvimento rigoroso de estudos que visem a futura aplicabilidade em ensaios clínicos.

O rigor do desenho experimental é essencial para reduzir a probabilidade de erro nas conclusões. Existem potenciais origens para erros nos estudos desenhados, podendo esses erros ser diminuídos adotando métodos como a randomização, ocultação e descrição rigorosa da metodologia. Numa revisão recente, observou-se que cerca de 70% dos estudos em animais não eram randomizados, cerca de 85% não tinham um método definido para alocação e em cerca de 65% a análise das variáveis não era realizada em ocultação [60]. Uma das estratégias para melhorar o rigor dos estudos pré-clínicos foi a introdução das ARRIVE guidelines [58]. Estas guidelines consistem numa check-list de 20 itens, contendo a informação considerada mínima e obrigatória para qualquer estudo em animais. Esta informação deve especificar para cada estudo: espécie, sexo, idade e detalhes sobre o tipo de alimentação dos animais, métodos experimentais e estatísticos. Deve incluir também uma secção orientada para o desenho do estudo (randomização, alocação e ocultação). Todos estes itens têm como objetivo
aumentar o rigor, a transparência e a compreensão do estudo e dos resultados. Por sua vez, para melhorar a qualidade dos ensaios clínicos em humanos, existem guidelines desde 1996 com modelos como o SPIRIT (Standard Protocol Items: Recommendations for Interventional Trials) [61] e o CONSORT (Consolidated Standards of Reporting Trials) [62].

A utilização de um grupo controlo deve fazer parte do desenho experimental a adotar. A ATM, sendo uma articulação bilateral dependente, onde vários estudos mostraram que uma intervenção influencia o lado contralateral [47,63], merece reflexão sobre como introduzir o controlo no estudo. Com efeito, vários estudos utilizaram o lado contralateral como controlo [53,64–66], introduzindo uma possível interpretação errada dos resultados. Assim, outras estratégias deverão ser adotadas, a fim de remover as referidas incertezas. Por último, a seleção das variáveis a estudar é, frequentemente, tema de debate. Para além das variáveis convencionalmente utilizadas, histológica [56,67,68] e radiológica [69], foi desígnio do presente trabalho propor a análise do tempo de mastigação e da cinemática ruminatória do modelo animal proposto. Não tendo conhecimento de estudos anteriores, que tenham avaliado o impacto de alterações na ATM na cinemática ruminatória e na mastigação, será importante perceber se esta avaliação é exequível e reprodutível em estudos futuros.
CAPÍTULO 2. OBJETIVOS DO ESTUDO

O principal objetivo desta dissertação foi avaliar o efeito da discectomia e discopexia da ATM, e analisar o seu impacto histológico, radiológico, no tempo de mastigação e na cinemática ruminatória da ovelha Black Merino.

Assim, foram definidos como objetivos específicos:

1. Definir o modelo animal apropriado para estudos pré-clínicos na área da ATM.
2. Caracterizar com precisão a anatomia e biomecânica do disco nativo da ovelha Black Merino.
3. Elaborar um desenho de estudo de investigação pré-clínico orientado à área da ATM, respeitando as ARRIVE guidelines.
4. Aplicar o modelo animal da ovelha Black Merino no desenho de estudo proposto.
5. Propor e testar variáveis piloto na área da cinemática ruminatória e tempo de mastigação e avaliar o seu impacto após discectomia bilateral e discopexia bilateral na ovelha Black Merino.
6. Avaliar o impacto histológico e imagiológico da discectomia bilateral e discopexia bilateral na ovelha Black Merino.
CAPÍTULO 3. DEFINIR MODELO ANIMAL PARA ESTUDOS PRÉ-CLÍNICOS NA ÁREA DA ARTICULAÇÃO TEMPOROMANDIBULAR

Trabalho 1

Animal model for temporomandibular joint research: morphological, histological and biomechanical characterization of the Black Merino sheep joint disc

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Animal model for temporomandibular joint research: morphological, histological and biomechanical characterization of the Black Merino sheep joint disc

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KEYWORDS
Sheep; Temporomandibular joint; Anatomy; Histology; Biomechanical characterization
SUMMARY

Preclinical trials are essential to the development of scientific technologies. Remarkable molecular and cellular research has been done using small animal models. However, significant differences exist regarding the articular behavior between these models and humans. Thus, large animal models may be more appropriate to perform trials involving the temporomandibular joint (TMJ). The aim of this work was to make a morphological (anatomic dissection and white light 3D scanning system), histological (TMJ in bloc was removed for histologic analysis) and biomechanical characterization (tension and compression tests) of sheep TMJ comparing the obtained results with human data. Results showed that sheep processus condylaris and fossa mandibularis are anatomically similar to the same human structures. TMJ disc has an elliptical perimeter, thinner in the center than in periphery. Peripheral area acts as a ring structure supporting the central zone. The disc cells display both fibroblast and chondrocyte-like morphology. Marginal area is formed by loose connective tissue, with some chondrocyte-like cells and collagen fibers in diverse orientations. Discs obtained a tensile modulus of 3.97 ± 0.73 MPa and 9.39 ± 1.67 MPa, for anteroposterior and mediolateral assessment. The TMJ discs presented a compressive modulus (E) of 446.41 ± 5.16 MPa and their maximum stress value (σ max) was 18.87 ± 1.33 MPa. Obtained results suggest that these animals should be considered as a prime model for TMJ research and procedural training. Further investigations in the field of oromaxillofacial surgery involving TMJ should consider sheep as a good animal model due to its resemblance of the same joint in humans.
INTRODUCTION

To improve human health, scientific discoveries and technologies must be translated into practical applications. Such advances classically begin with basic research and then progress to the clinical level. Inherent to the development of new technologies is the role of preclinical trials using animal models. Although no animal model can fully replicate human conditions, animal models are key for the evaluation of mechanisms of disease, testing new technologies and applying new procedures.

Temporomandibular joint (TMJ) is the most frequently used joint in the human body. TMJ opens and closes 1500-2000 times daily and is essential for everyday functions of the mouth such as mastication, speech, deglutition, yawning and snoring involving special mandatory synergy of both articular sides [10]. Joint surfaces are convex and, therefore, smooth joint movements are only possible due to an intra-articular disc between them. TMJ disc is an essential component in the normal TMJ and has the following functions: it distributes the intra-articular load, stabilizes the joints during translation and decreases the wear of the articular surface [70,71]. TMJ disc displaced, malformed or damaged, can induce pathologic processes of internal derangement and/or osteoarthritis [72,73]. Currently patients suffering from severe temporomandibular dysfunction (TMD) have few treatment options. Without safe, effective TMJ disc implants, many patients undergo disectomy: a surgical procedure that removes the injured TMJ disc aiming to reduce severe TMD symptoms. This procedure may not be the ideal as the TMJ is left without an important functional structure. Since the previous problems associated with alloplastic materials used to substitute TMJ disc such as silicone and Proplast-Teflon (PTIPI, Vitek, Inc, Houston, Texas, USA) [74,75], many groups discarded investigation in this field. However, the potential impact of a synthetic temporomandibular interpositional implant (TII) is immense. Failures of the synthetic TII have generally been attributed to the lack of knowledge concerning the TMJ biomechanical and biochemical aspects. The development of new technologies for scaffolds engineering regarding TMJ disc is growing [65,76–78] and the ideal animal model for TMJ research should be well characterized. The choice of an animal for experimental design is not straightforward. Due to physiological and anatomical differences between the human TMJ and that of experimental animals, there is no animal model that is valid per se. TMJ is a cardinal feature that defines the class Mammalia and separates
mammals from other vertebrates [79]. TMJ shows remarkable morphological and functional variation between different species, reflecting not only the great mammalian adaptation to feeding mechanisms but also different biomechanical behavior [80]. The morphological variations are either correlates of loading (e.g. size of articular surfaces) or movement (e.g. orientation of the joint), or both. Loading of the TMJ is a reaction force arising from the contraction of masticatory muscles; its magnitude depends strongly on the position of the bite point relative to the muscle action line [81].

Many commonly used laboratory animals, especially rodents, fall in the category of minimal TMJ loading, especially during chewing. In contrast, carnivores such as dogs sustain TMJ loads that are higher than those of primates [82]. Opening of the jaw usually involves a combination of rotation and forward sliding (translation), but some carnivores have lost the ability to slide and some specialized anteaters instead use a rotation around the long axis of the curved mandible [80]. The most extreme evolutionary variants include:

- loss of the synovial cavity in some baleen whales;
- loss (or possibly primitive absence) of the disc in monotremes, some marsupials, and some edentates (anteaters and sloths);
- variations in the orientation of the joint cavity from sagittal (many rodents) to transverse (many carnivores);
- reversal of the usual convex/concave relationship so that the processus condylaris becomes the female element (many artiodactyl ungulates such as sheep and cattle).

In addition, the relative size of the joint is exceedingly variable. Sheep, rabbit and monkey have been used as TMJ disc defect models in many studies [83–90]. Monkey model is barely used in recent years, considering the high cost, difficult surgical operation and ethical approval. Rabbit is an excellent option for TMJ disc anterior dislocation studies but the small size of TMJ increases the difficulty for surgical approach and disc manipulation. The authors agree with others studies considering sheep is a valid option for TMJ studies due to TMJ size, processus condylaris and fossa mandibularis shape, disc size, morphology and attachments [79]. However, a deep biochemical and biomechanical characterization of the sheep TMJ is lacking in the available literature. Hence, the aim of the present study was to
examine the morphological, histological and biomechanical properties of TMJ discs extracted from sheep (*Ovis aries*). It was hypothesized that these discs would present high similarity with available data on human TMJ.

**Materials and methods**

The material used for this study was obtained from sheep slaughtered for meat consumption. A total of 15 heads from *Black Merino* female sheep, 40 to 50kg, were used: 6 for morphological characterization, 4 for histological characterization and 5 for biomechanical testing. One of the major requirements for this study was to use fresh TMJ discs; for that reason a team of certified surgeons was available 5 days weekly to collect fresh TMJ up to a maximum of 5 hours after death. Regarding the animal ethical considerations, the present study design was approved by the Portuguese National Authority for Animal Health.

**Morphological characterization**

For morphological characterization 12 fresh TMJ discs were collected from six sheep heads. A surgical discectomy was performed exposing and identifying TMJ anatomical structures. All muscular attachments were removed to obtain clean TMJ discs. Discs were submersed for 5 minutes in a *ColorBond* solution, an extremely fast curing infiltrant, designed to rapidly strengthen 3D-printed parts. This submersion was essential to maintain the correct morphology for the 3D scanning. A white light 3D scanning system (Steinbichler — COMET 5®) and the appropriate software were used to replicate the discs in a 3D virtual model. Once the discs removed, two of the skulls were boiled in water (120 °C) for 2 h to allow the procurement of complete clean crania.

**Histological characterization**

Four sheep heads were used to conduct the histological investigation. The TMJ were removed using a necropsy bone oscillatory saw according to the following anatomic references: cranial — cranial aspect of processus coronoideus in the section of the arcus zygomaticus; caudal — external to the meatus acusticus. The dorsal reference was established to the squamous temporal bone. The ventral reference was 2 cm ventral to the meatus acusticus in the zone of angulus
Capítulo 3. Definir Modelo Animal para Estudos Pré-clínicos na área da Articulação Temporomandibular

The joints were fixed in 10% buffered formalin for ten days. Decalcification was obtained by immersion in 10% formic acid for three weeks, after which the articulations were cut sagittally and transversally through the whole processus condylarisis. After intensive washing the fragments were submitted to routine tissue processing with paraffin embedding. Four-micron sections were stained with hematoxylin and eosin (H&E) and with Orcein to show elastic fibers in the disc. Digital images were obtained with an Olympus DP21 camera.

Biomechanical testing

Five sheep heads were used for biomechanical studies. TMJ discs were removed and immersed in a saline solution for transport up to the bioengineering facilities (1 hour maximum). All muscular attachments and ligaments were removed to obtain a clean fibrocartilaginous disc. Ten clean discs were obtained but one was excluded due to surgical damaging. Consequently, 9 discs were randomized in 3 groups and tested in different mechanical tests: Tensile modulus (E), tensile strength and elongation were tested in: anteroposterior tests (APT) and mediolateral tests (MDT). Compression tests (CT) were performed using a stress-strain tests. In case of anteroposterior tensile test, during loading, the TMJ discs were stretched in the direction represented on Fig. 1A, while in mediolateral tensile test the direction of stretching was as shown on Fig. 1B.

**Figure 1.** Direction of loading on: (A) anteroposterior and (B) mediolateral tensile tests. The dotted line represents the limit used to fix temporomandibular joint (TMJ) discs in grips. Tension tests used a strain rate of 0.5 mm/min with an initial distance between grips (L0) of 2 mm. All tests were conducted on a Zwick Z100 strength-testing machine (Zwick GmbH & Co. kg, Germany) equipped with a 10 kN load cell. For the compression tests the same rate was applied.
Results

Morphological characterization

In the sheep heads studied, the TMJ was located, as expected, in the posterior segment of the side of the face, cranoventral to the external meatus acusticus, being a diarthrodial, bicondylar joint that allows normal opening and closing of the mandible. It comprised the superior articulating face, the fossa mandibularis of temporal bone, and the processus condylaris, as the inferior articulating surface (Figs. 2 and 6). A protruding processus coronoideus was noted (Fig. 2).

Figure 2. Right view of a sheep skull used in the present study. (1) Processus condylaris, (2) fossa mandibularis, (3) arcus zygomaticus, (4) eminentia articularis, (5) external meatus acusticus, (6) processus mastoideus, (7) collum mandibulae. P: posterior; A: anterior; S: superior; I: inferior.

The superior articulating surface (fossa mandibularis) was located in the inferior zone of temporal bone, lateral of foramen ovale and anterior to the external meatus acusticus. The fossa mandibularis was anteroposterior larger than mediolateral with a convexity downwards. The inferior articulating surface (Fig. 3) is represented by the processus condylaris, with ellipsoidal shape with the longer axis in the mediolateral position, the mean measures being 23.47 mm long (σ=0.87) and 8.32mm wide (σ=1.54). The processus condylaris was mediolateral concave. The fossa mandibularis receives the processus condylaris. With an easy surgical
approach the authors located the fibrocartilaginous joint disc interposed between the fossa mandibularis and the processus condylaris (Fig. 4).

**Figure 3.** Articular surfaces of the temporomandibular joint (TMJ). A. Superior articular surface: (1) fossa mandibularis, (2) arcus zygomaticus, (3) foramen ovale. B. Inferior articular surface: (1) processus condylaris, (2) processus coronoideus, (3) incisura mandibulae, (4) foramen mandibulae. P: posterior; A: anterior; M: medial; L: lateral.

**Figure 4.** View of the right temporomandibular joint (TMJ). To improve visualization the authors pulled down the processus condylaris. (1) Cartilage surface of fossa mandibularis in the upper joint compartment, (2) temporomandibular joint disc, (3) retrodiscal tissue, (4) muscle pterygoideus lateralis (5) cartilage surface of the processus condylaris (6) external meatus acusticus. P: posterior; A: anterior; M: medial; L: lateral.
This disc separates an upper joint cavity from a lower one. The first was consistently larger than the second. The bony structures were coated with cartilage more evident in the processus condylaris. In the ewes studied, the joint disc had an elliptical shape, being substantially thinner in the center than at the periphery. TMJ disc regions are commonly classified as anterior band, posterior band, and intermediate zone (Fig. 5). The intermediate zone exhibits differences from its lateral to medial aspects, being often subdivided into lateral, medial and central region. The bands discs are thicker than the intermediate zone. The mean length and width of the 12 analyzed fresh TMJ discs were 21.23 mm ($\sigma = 1.53$) and 11.49 mm ($\sigma = 0.62$), respectively. Anterior and posterior band thicknesses were 1.05mm ($\sigma = 0.07$) and 1.27mm ($\sigma = 0.04$), respectively. Mean central thickness was 0.76 mm ($\sigma = 0.09$). The same measures obtained from the 3D virtual models were totally similar to the ones registered in the fresh discs. An important report and consistent with all TMJ was the presence of viscous fluid in upper and lower compartment. This fluid was not analyzed.

Figure 5. Temporomandibular joint (TMJ) disc. A. Fresh disc with attachments. B. Fresh disc without attachments. C. TMJ disc submitted to ColorBond treatment: (1) anterior band, (2) posterior band, (3) medial band, (4) lateral band. D. TMJ disc 3D virtual model.
Histological characterization

The histological study of the sheep TMJ revealed that the articular disc was attached anteriorly and posteriorly to the articular capsule composed by fibrous tissue. Both the fossa mandibularis and the processus condylaris surfaces were covered by a fibrocartilaginous layer. However, the fibrocartilaginous layer covering the processus condylaris was considerably thicker than the layer covering the fossa mandibularis (Fig. 6). The central thin part of the disc consisted of scattered fibroblasts and densely packed, thick collagen fiber bundles arranged mainly in an anteroposterior direction. The collagen fibers were not straight but showed evidence of a wavy outline. The anterior and posterior disc portions were in turn occupied by collagen fiber bundles with diverse orientations (Fig. 7). In some areas, these two portions showed chondrocyte-like cells residing in lacunae distributed among less compact collagen fibers (Fig. 7). Each lacuna was surrounded by minimal amount of amorphous matrix. The posterior band blended, in the retrodiscal space, with loose connective tissue with profuse blood and nerve supply. A few small caliber blood vessels, surrounded by loose connective tissue, were observed in all parts of the disc (Fig. 7). Also occasional unilocular adipocytes were present at both the anterior and posterior attachments of the disc. Orcein-positive elastic fibers were found throughout the disc, being apparently more abundant in the thinnest central portion. In this disc area, elastic fibers were arranged mostly in parallel to the collagen bundles (Fig. 8). Instead, in the anterior and posterior disc portions, elastic fibers showed a reticular distribution among collagen fibers and chondrocyte-like cells (Fig. 8).
Figure 6. Microscopic overview of a sagittal section of the temporomandibular joint (TMJ) stained with haematoxylin- eosin. T: temporal bone; D: central region of the intermediate area of the joint disc; M: processus condylaris (bar = 10 µm).
Figure 7. Photomicrographs of various regions of the sheep temporomandibular joint (TMJ) disc stained with haematoxylin-eosin. A. Tightly packed collagen fibers with parallel arrangement interspersed by fibroblasts in the central portion of the TMJ disc (bar = 50 µm). B. Haphazardly arranged collagen fiber bundles in the posterior band of the TMJ disc (bar = 50 µm). C. Chondrocyte-like cells in the anterior band of the TMJ disc (∗200, bar = 50 µm). D. Small caliber blood vessels (arrows) in the TMJ disc (∗100, bar = 100 µm).

Figure 8. Photomicrographs of the central zone (A) and (B) anterior band of sheep temporomandibular joint (TMJ) disc stained with orcein for detection of elastic fibers (bar = 50 µm). A. Longitudinal elastic fibers follow the wavy structure of collagen bundles. B. Loose mesh elastic fibers distributed between chondrocyte-like cells.
Biomechanical characterization

In Table 1, the measures of the discs used in the mechanical tests are presented. Tensile tests performed revealed that TMJ discs presented different behaviors for anteroposterior and mediolateral directions (Fig. 9). The obtained results demonstrated that the tensile modulus of mediolateral tensile tests is higher than anteroposterior tensile tests, as well as the tensile strength and elongation at break (Figs. 10 and 11). In Table 2 the results obtained for the tested discs for tensile modulus, tensile strength and elongation at break are summarized.

### Table 1. Length, with and thickness of 9 discs tested.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dimensions (mm)</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length</td>
<td>Width</td>
</tr>
<tr>
<td>APT1</td>
<td>22.71</td>
<td>11.06</td>
</tr>
<tr>
<td>APT2</td>
<td>23.89</td>
<td>10.69</td>
</tr>
<tr>
<td>APT3</td>
<td>20.43</td>
<td>11.29</td>
</tr>
<tr>
<td>MDT1</td>
<td>19.60</td>
<td>12.63</td>
</tr>
<tr>
<td>MDT2</td>
<td>20.57</td>
<td>10.56</td>
</tr>
<tr>
<td>MDT3</td>
<td>20.05</td>
<td>11.39</td>
</tr>
<tr>
<td>CT1</td>
<td>20.75</td>
<td>10.07</td>
</tr>
<tr>
<td>CT2</td>
<td>20.49</td>
<td>11.93</td>
</tr>
<tr>
<td>CT3</td>
<td>19.94</td>
<td>10.44</td>
</tr>
</tbody>
</table>

APT: anteroposterior tests; MDT: mediolateral tests; CT: compression tests.

Mechanical testing under compression was performed to evaluate the macro-mechanical performances of the TMJ discs. Fig. 12 demonstrates the compressive stress-strain curves of the tested discs. The TMJ discs presented a compressive modulus (E) of 446.41±5.16MPa and their maximum stress value (σ max) was 18.87 ± 1.33 MPa.

### Table 2. Mechanical tensile properties of TMJ discs.

<table>
<thead>
<tr>
<th>Tensile test</th>
<th>Tensile modulus E (MPa)</th>
<th>Tensile strength (MPa)</th>
<th>Elongation at break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anteroposterior</td>
<td>3.97 ± 0.73</td>
<td>4.34 ± 1.22</td>
<td>170.92 ± 47.87</td>
</tr>
<tr>
<td>Mediolateral</td>
<td>9.39 ± 1.67</td>
<td>13.21 ± 0.85</td>
<td>195.23 ± 20.44</td>
</tr>
</tbody>
</table>

TMJ: temporomandibular joint. Tensile modulus (E), tensile strength and elongation at break are reported as mean value ± standard deviation.
Figure 9. Tensile mechanical performance of temporomandibular joint (TMJ) discs in anteroposterior and mediolateral directions.

Figure 10. Mediolateral tensile mechanical performance of temporomandibular joint (TMJ) discs. Tension tests used a strain rate of 0.5 mm/min with an initial distance between grips (L0) of 2 mm. All tests were conducted on a Zwick Z100 strength-testing machine equipped with a 10 kN load cell.
Figure 11. Anteroposterior tensile mechanical performance of temporomandibular joint (TMJ) discs. Tension tests used a strain rate of 0.5 mm/min with an initial distance between grips (L0) of 2 mm. All tests were conducted on a Zwick Z100 strength-testing machine equipped with a 10 kN load cell.

Figure 12. Compressive mechanical performance of temporomandibular joint (TMJ) discs. Compression tests used a compression rate of 0.5 mm/min.
Discussion

TMJ disc is a specialized fibrocartilaginous tissue, located between the processus condylaris and the fossa mandibularis [70,75,91] as shown in our sheep morphologic characterization. In humans TMJ disc has an elliptical perimeter, thinner in the center than on periphery. Disc periphery acts like a ring structure supporting the central zone. The same was observed in sheep disc morphology. The functions of the TMJ disc are:
• to improve the fit between bony surfaces;
• to provide stability during mandibular movements;
• to distribute masticatory forces [92].
This capacity is due to the high concentration of collagen fibers. This ring structure around the disc is an important structural aspect to support disc connections. The connection area is rich in elastic fibers, which is essential to disc mobility in the joint.
As it was shown in the morphological characterization of the sheep TMJ, this anatomical structure revealed several similar characteristics with the TMJ in humans, including the mediolateral diameter being longer than the anteroposterior, the long axis of the processus condylaris directing backwards, and larger anterior condylar slope. One of the main differences is the concave form of the mediolateral processus condylaris that is convex in humans. The processus condylaris forms a small anteroposterior and mediolateral depression to fit exactly in the fossa mandibularis, unlike the human processus condylaris, which is rounded anteroposterior and mediolateral. The fossa mandibularis is anteroposterior larger than mediolateral with a convexity downwards contrarily to the fossa mandibularis in humans that is concave upwards. The fossa mandibularis allows for the free mediolateral movement of the processus condylaris for rumination. The articular tubercle, a special feature in humans, is rudimentary in the sheep, since the path of the processus condylaris movement is mediolateral, contrarily to the one in humans, which is mostly anteroposterior. Comparatively, the fossa and processus condylaris of the sheep are much like edentulous human TMJ, much flatter. Architecturally, the processus condylaris in both species also has a thin external cortex that surrounds the medullary bone that is made up of trabecular bone. There is also a thin layer of fibrocartilage covering the condylar surface and entire fossa mandibularis, indicating
parts of the temporomandibular joint that are subject to highest loading. TMJ relation with the external acusticus meatus, foramen ovale and the joint disc position interposing processus condylaris and fossa mandibularis are similar to human TMJ anatomy. TMJ disc morphology is very similar to human TMJ disc. The choice of sheep as an animal model for TMJ studies has been used for several years [83–90]. TMJ disc implants can be an efficacious complement in bioengineered joint reconstruction and animal models may offer the possibility to conduct informative preclinical studies. One of the most important problems to create an effective TII is to replicate the biomechanics characteristics of the native disc. Therefore, information on the biomechanical properties of the substitute material is indispensable for further investigation in TMJ disc tissue engineering. During mandibular movements the TMJ disc is subject to a multitude of different loading regimens. TMJ disc behaves as a viscoelastic structure acting as a stress absorber and a stress distributor [92–94]. Elastic fibers play an important role providing the disc with the necessary viscoelastic structure. During every type of loading, the disc undergoes a deformation, while internal forces are produced within the tissue [91]. The internal forces are quantified by the amount of stress, which is defined as force per unit area in Pa (1 Pa = 1 N/m²). There are only two studies available on bovine TMJ disc in which tensile and compressive modulus have been compared using the same experimental protocol and material [91,95]. In these studies tensile modulus ranged between 22 and 26 MPa, and compressive modulus between 14 and 17 MPa. Data on the porcine, canine and human TMJ discs are available in the literature but methods used for disc obtainment and processing are not always clear. Reported tensile modulus are approximately 0.5—80 MPa, 20—25 MPa and 40—100 MPa, respectively, for the referred above animal models [91,96,97]. In order to evaluate the mechanical behavior of the sheep TMJ discs, the authors report, for the first time as it was possible to estimate, anteroposterior and mediolateral tensile modulus and compressive modulus. The use of fresh TMJ discs have contributed for the results to be representative of reality. Sheep mandibular movements are mostly mediolateral explaining the better performance of TMJ disc supporting tension in the mediolateral direction. In conclusion, sheep seems to be an excellent experimental model for TMJ studies, being a large species with many anatomical similarities to the human structure in relation to surgical approach, anatomical
structures size, shape and position of the processus condylaris. TMJ disc seems to be very similar human TMJ disc concerning morphology, histology and biomechanics. It is the author’s purpose that the present work will help further research in the field of oromaxillofacial conducted in sheep as an excellent alternative to other more conventional experimental animal species, also more suitable for procedural surgical training.
Trabalho 2

Novel approach for 3D virtual model of TMJ disc morphology. Preliminary results


Novel approach for 3D virtual model of TMJ disc morphology. Preliminary results

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ABSTRACT

**Introduction:** Regenerative medicine is an immense field with extreme and challenging obstacles. The first challenge is to understand the morphological, histological, biochemical and biomechanical characteristics of the structure to reproduce. This multitask and multidisciplinary approach is essential to determine the success of regenerative medicine. The authors present a new method to reproduce 3D morphology of anatomical structures with sheep TMJ disc as an example.

**Objective:** The main objective of the authors was to reproduce a 3D virtual model of six sheep TMJ disc.

**Methods:** A medical surgeon performed surgical discectomy of TMJ disc in fresh sheep cadaver with microscope. The second step was related to remove all disc muscular attachments to obtain a clean cartilage disc. The disc was submersed in a solution to maintain the correct morphology. With a white light 3D scanning system and appropriate software we reproduce the morphology of six sheep TMJ disc.

**Conclusion:** 3D virtual model of TMJ disc were success- fully reproduced using a white light 3D scanning system. This technique has economic and time related advantages. For precision and detailed results we need to conduct more studies.
Trabalho 3

Bioengineered Temporomandibular Joint Disk Implants: Study Protocol for a Two-Phase Exploratory Randomized Preclinical Pilot Trial in 18 Black Merino Sheep (TEMPOJIMS)

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KEYWORDS
temporomandibular joint disorders (TMD); temporomandibular joint bioengineered disk implants; temporomandibular randomized preclinical trial protocol
ABSTRACT

Background: Preclinical trials are essential to test efficacious options to substitute the temporomandibular joint (TMJ) disk. The contemporary absence of an ideal treatment for patients with severe TMJ disorders can be related to difficulties concerning the appropriate study design to conduct preclinical trials in the TMJ field. These difficulties can be associated with the use of heterogeneous animal models, the use of the contralateral TMJ as control, the absence of rigorous randomized controlled preclinical trials with blinded outcomes assessors, and difficulties involving multidisciplinary teams.

Objective: This study aims to develop a new, reproducible, and effective study design for preclinical research in the TMJ domain, obtaining rigorous data related to (1) identify the impact of bilateral discectomy in black Merino sheep, (2) identify the impact of bilateral discopexy in black Merino sheep, and (3) identify the impact of three different bioengineering TMJ discs in black Merino sheep.

Methods: A two-phase exploratory randomized controlled preclinical trial with blinded outcomes is proposed. In the first phase, nine sheep are randomized into three different surgical bilateral procedures: bilateral discectomy, bilateral discopexy, and sham surgery. In the second phase, nine sheep are randomized to bilaterally test three different TMJ bioengineering disk implants. The primary outcome is the histological gradation of TMJ. Secondary outcomes are imaging changes, absolute masticatory time, ruminant time per cycle, ruminant kinetics, ruminant area, and sheep weight.

Results: Previous preclinical studies in this field have used the contralateral unoperated side as a control, different animal models ranging from mice to a canine model, with nonrandomized, nonblinded and uncontrolled study designs and limited outcomes measures. The main goal of this exploratory preclinical protocol is to set a new standard for future preclinical trials in oromaxillofacial surgery, particularly in the TMJ field, by proposing a rigorous design in black Merino sheep. The authors also propose to test the feasibility of pilot outcomes.
INTRODUCTION

The temporomandibular joint (TMJ) is the most frequently used joint in the human body. The TMJ opens and closes 1500 to 2000 times daily and is essential for everyday functions of the mouth, such as mastication, speech, deglutition, yawning, and snoring, involving special mandatory synergy of both articular sides [10]. The TMJ disk is an essential component in the normal TMJ and has the following functions: (1) it distributes the intra-articular load, (2) it stabilizes the joints during translation, and (3) it decreases the wear of the articular surface [70,71]. The majority of TMJ disorders (TMD) are successfully treated with reversible, conservative, and low-tech treatments such as education and counseling, therapeutic exercises, splint therapy, and pharmacotherapy [98,99]. When the TMJ disk is displaced, malformed, or damaged, it can induce serious internal pathologic processes and/or osteoarthritis [72,73]. Currently, patients suffering from severe TMD have limited validated treatment options. Most surgical approaches, such as TMJ discectomy, do not restore the structural or biological properties of the articulation and disk. This procedure may not be ideal because the TMJ is left without an important functional structure. A variety of interpositional materials have been used to replace the removed disks, including synthetic materials manufactured from silicone, teflon, polytetrafluoroethylene, and biological interpositional grafts taken from different anatomic sites [74,100–102]. These interpositional materials do not take in consideration the anatomy and biochemical and biomechanical characteristics of the TMJ native disk [103], and some of them have been associated with serious complications for the patients [102–104]. In the late 1980s, Proplast/Teflon TMJ (synthetic interpositional implant) were found to be harmful in many patients. The breakdown of the material, probably caused by TMJ high biomechanical forces, lead to fragmented particles that resulted in an immune foreign body response that caused problems ranging from severe cutaneous inflammatory reaction in the preauricular and cheek areas [105] to severe degenerative joint disease with perforation into the middle cranial fossa [106,107]. The result was a dramatic clinical spectrum of failures for these implants [74]. In December 1991, the US Food and Drug Administration’s Bulletin recommended immediate removal of all previous TMJ Proplast/Teflon implants because of the
mechanical failures, many resulting in progressive bone degeneration [108]. In a 1992 workshop, the American Academy of Oral and Maxillofacial Surgery instructed the discontinuation of Proplast/Teflon [108]. The absence of efficacious options to substitute the TMJ disk can be related to difficulties in the translation of animal evidence to the clinical practice in humans. These limitations are likely related to:

1. the use of heterogeneous animal models with conflicting results, possibly due to variable anatomy and intra-articular loading between species [79,109];
2. the use of the contralateral TMJ as control, which may be associated with contralateral overloading [110];
3. the biomaterials used to replace the disk do not account for the morphologic and biomechanical characteristics of the native disk;
4. absence of randomized controlled trials with blinding of outcomes’ assessors; and
5. lack of multidisciplinary teams involved in the project.

Preclinical research should promote the effective translation of knowledge into practice. The previously mentioned aspects can limit the effective translation of quality scientific knowledge into clinical practice and these may present potential issues to patients, clinicians, and scientific progress. The contemporary absence of successful options to substitute the TMJ disk is still a major issue for public health. Little has changed in the past decade regarding study designs for TMJ investigation, and the treatment for patients with severe TMD remains controversial. The main objective of the Temporomandibular Joint Interposai Material Study (TEMPOJIMS) is to develop a new, reproducible, and effective study design for preclinical research in the TMJ field. The second goal is to progress in bioengineering and regenerative medicine evaluating the benefits of a TMJ bioengineering implant to substitute the damaged native TMJ disk. This preclinical exploratory study is divided into two phases. Phase 1 of this study is a blinded randomized preclinical trial, designed to investigate if the TMJ undergoes important injury in bilateral discectomy, bilateral discopexy, and sham surgery. Phase 2 intentions are to evaluate the safety and efficacy of three different TMJ bioengineering implants using the same rigorous method of phase 1.
METHODS

Study Design
The TEMPOJIMS is a two-phase exploratory randomized controlled preclinical trial planned to gather preliminary information to (1) evaluate a new study design for TMJ investigation; (2) evaluate the black Merino sheep animal model for TMJ investigation; (3) evaluate TMJ behavior under bilateral surgical intervention (discectomy and discopexy) using a histologic primary outcome (microscopic scoring of destructive changes in TMJ using a modified Mankin scoring system [111]), secondary imaging outcome (imaging scoring of TMJ); (4) testing the applicability of pilot secondary outcomes predominantly for ruminant kinetics; and (5) obtain a baseline for interpretation of TMJ disk bioengineering implants results. Phase II is aimed to test safety and efficacy of three different bilateral TMJ bioengineering disk implants (Figure 1). Outcome evaluators and analysts are blinded for surgical assessments.

Figure 1. Study design.

Major institutions involved in this study are (1) Lisbon Faculty of Medicine for study design, coordination, and statistical analysis; (2) Interdisciplinary Centre of Research in Animal Health in Faculty of Veterinary Medicine for histological preparation and veterinary support of all animals; (3) Centre for Rapid and
Sustainable Product Development for bioengineered disk implants (disks I and II); (4) Bioengineering, Surgery, Chemical Engineering, Mechanical Engineering and Materials Science, University of Pittsburgh, for bioengineered disk implants (disk III); (5) Department of Oral and Maxillofacial-Head and Neck Surgery, University Hospital Infanta Cristina, Badajoz, Spain, for surgical support; (6) Institute of Bone and Joint Research-Northern Sydney Local Health District-Sydney Medical School Northern, University of Sydney, Australia, for histological analysis; and (7) Radiology Department of Santa Maria Hospital, Lisbon, Portugal, for imaging analysis.

**Animal Model**

A variety of strains/breeds of sheep have been used in TMJ investigations. To decrease biological variability, the authors recommended black Merino sheep as the animal model to conduct the study [109]. As recommended, the authors proposed to use “sheep skeletally mature” at ≥2 years of age [112]. The inclusion criteria are certified black Merino sheep, adult (age 2-5 years), female, and in good health condition (veterinary check-up is performed on all animals). Regarding the animal ethical considerations, the study design was approved by the Portuguese National Authority for Animal Health registered with number 026618. The study design and organization respect the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

**Baseline and Follow-Up Evaluation**

The baseline and follow-up evaluations are outlined at particular time points (Figure 2). Pilot secondary outcomes and weight are measured at days 11, 10, and 9 before surgery (details on secondary outcomes are reported in outcomes measures). Transportation to surgical facilities is performed 5 days before surgery to avoid animal stress and allow familiarization to the temporary facilities. Head computerized tomography (CT) scan is performed on the day of surgery taking advantage of pre anesthesia sedation. Ten days after surgery, animals are transported to TEMPOJIMS main facilities. Days 19, 20, and 21 after surgery, the follow-up secondary outcomes start to be recorded every 30 days for 6 months (Figure 2). At the end, animals are sacrificed and a new CT scan is performed to measure the imaging outcome and to begin the histologic preparation.
Randomization, Allocation, and Blinding

The randomization is performed by a statistical group not involved in the outcome assessments, managed by Lisbon Faculty of Medicine. Allocation to each randomized group is performed preoperatively by sealed envelope and separately for phase 1 and phase 2 of the study. The surgical team is not blinded to treatment allocation given the type of intervention; however, surgical team members are not involved in outcome assessments. All outcome evaluators are blinded to intervention. In phase 1, 10 sheep are allocated to the intervention group: sham surgery group (n=3), discectomy group (n=3), discopexy group (n=3), and backup group (n=1). The backup sheep is planned to be used if death occurs due to anesthesia or another complication not related to the surgical intervention. In phase 2, 10 sheep are randomly assigned to disk I group (n=3), disk II group (n=3), disk III group (n=3), and backup group (n=1) (Figure 1).

Intervention Phase

Anesthesia Protocol

Fasting and water restriction are required 24 hours before surgery. Sedation is performed with diazepam (0.5 mg/kg iv), followed by anesthesia induction with ketamine (5 mg/kg iv). Oral intubation is performed and anesthesia is maintained with isoflurane (1.5% to 2%). To assure animal analgesia, meloxicam (0.5 mg/kg iv,
bid) is administered on surgery day and during 4 days postoperatively. Antibiotic prophylaxis with amoxicillin and clavulanic acid are used for 5 days.

**Surgical Intervention Protocol for Phases 1 and 2**

**Phase 1**

Bilateral discectomy (n=3): under general anesthesia, the surgical team perform a preauricular skin incision and a blunt dissection of the soft tissue covering the joint. The joint area is disclosed and the articular capsule is incised. The disk and its attachments are identified. The medial, anterior, posterior, and lateral disk attachments are detached and discectomy is performed. The wound is closed in layers.

Bilateral discopexy (n=3): under general anesthesia, the surgical team perform a preauricular skin incision and a blunt dissection of the soft tissue covering the joint. The joint area is disclosed and the articular capsule is incised. The disk and its attachments are identified. The lateral and posterior disk attachments are detached and sutured with poly- p-dioxanone (PDS) 3/0. The wound is closed in layers.

Sham surgery (n=3): under general anesthesia, the surgical team will perform a preauricular skin incision and a blunt dissection of the soft tissue covering the joint. The capsule is not incised. The wound is closed in layers.

**Phase 2**

Disk I (n=3): under general anesthesia, the surgical team perform a preauricular skin incision and a blunt dissection of the soft tissue covering the joint. The joint area is disclosed and the articular capsule is incised. The disk and its attachments are identified. The medial, anterior, posterior, and lateral disk attachments are detached and discectomy is performed. The disk I is introduced into the articular space and sutured in the lateral attachments. The wound is closed in layers. Disk I will be an alternative biomaterial and for intellectual reasons cannot be revealed in this paper.
Disk II (n=3): under general anesthesia, the surgical team perform a preauricular skin incision and a blunt dissection of the soft tissue covering the joint. The joint area is disclosed and the articular capsule is incised. The disk and its attachments are identified. The medial, anterior, posterior, and lateral disk attachments are detached and discectomy is performed. The disk II is introduced into the articular space and sutured in the lateral attachments. The wound is closed in layers. Disk II will be a porous poly(glycerol sebacate) (PGS) scaffold reinforced with polycaprolactone (PCL).

Disk III (n=3): under general anesthesia, the surgical team perform a preauricular skin incision and a blunt dissection of the soft tissue covering the joint. The joint area is disclosed and the articular capsule is incised. The disk and its attachments are identified. The medial, anterior, posterior, and lateral disk attachments are detached and discectomy is performed. The disk III is introduced into the articular space and sutured in the lateral attachment. The wound is closed in layers. Disk III will be a porous PGS scaffold prepared by a modified salt fusion method. Briefly, ground salt particles (150 mg) with a size range of 25 to 32 μm will be placed into a 3-D printed mold. The mold will be transferred to an incubator at 37°C and 90% relative humidity for 1 hour. The fused templates of salt particles will dry in a vacuum oven at 90°C and 100 millitorr (mTorr) overnight, removing salt cake carefully from the mold before further processing. Fresh-made PGS dissolved in tetrahydrofuran (THF; 20 wt%, 380 μL, salt:PGS=2:1) added to the salt cake, and the THF is allowed to evaporate completely in a fume hood for 30 minutes. The salt cake is transferred to a vacuum oven and cured at 150°C and 100 mTorr for 24 hours. The resultant PGS-impregnated salt templates are soaked in deionized water for 4 hours, and then replaced with water for 4 hours, with water exchange every 4 hours during the first 12 hours. After the 12-hour water bath, scaffolds are transferred to deionized water for another 24 hours with water exchange every 8 hours. The resultant scaffolds are frozen down at –80°C and then the lyophilization process is applied. Ten days for recovery is contemplated for wound care and postoperative medication (see Figure 2).


### Outcome Measures

The primary outcome is the microscopic scoring of destructive changes in the TMJ using a modified Mankin scoring system [111]. Secondary outcomes are imaging scoring of TMJ destructive changes, absolute masticatory time, ruminant time per cycle, ruminant kinetics, ruminant area, and sheep body weight. Primary and secondary outcome parameters are outlined in more detail in Figure 3.

**Primary outcome parameter**
- Histological gradation of TMJ degeneration with modified Mankin system [21]

**Secondary outcome parameter**
- Absolute masticatory time: According to baseline and follow-up planning, masticatory time for 150g dry pellets ingestion is registered
- Ruminant time per cycle: According to baseline and follow-up planning, 15 rumination cycles are recorded and time per cycle is registered
- Ruminant kinetics: Appropriate software is used to track rumination trajectories creating a trajectory average
- Ruminant area: Appropriate software is used to measure the area of rumination in pixels
- Weight: According to baseline and follow-up planning, weight is registered
- Imaging: According to baseline and follow-up planning, TMJ CT is performed to visualize degenerative changes

**Figure 3.** Primary and secondary outcome parameters.

### Primary Outcome

The goal is to evaluate histologic gradation of TMJ destructive changes. The time point is 6 months following surgical intervention.

Six months after surgery, the TMJ is removed using a necropsy bone oscillatory saw according to the following anatomic references: cranial (cranial aspect of coronoid process in the union region of the zygomatic process), caudal (external to acoustic meatus), dorsal (reference is established to the squamous temporal bone), and ventral (reference is fixed 2 cm below the acoustic meatus in the zone of stylohyoid angle). The joints are fixed in 10% buffered formalin for 24 hours and stored in 70% ethanol. Decalcification is obtained by immersion in 10% formic acid in 5% formalin for up to 20 days, after which the articulations are cut sagittally through the whole
condyle. After decalcifying, TMJ articulations are immersed in three graded methyl salicylate/paraffin mixtures and cut sagittally through the lateral into the central part of the TMJ. Histological sections are sent to Sydney Institute of Bone and Joint Research for histological scoring using a modified Mankin scoring system [111]. This assessment is performed and classified independent by two histologists who will be blinded to intervention. A third histologist will act as arbiter in case of disparity.

**Secondary Outcomes**

The features evaluated are imaging analysis, absolute masticatory time, ruminant time per cycle, ruminant kinematics, ruminant area, and sheep weight (see Multimedia Appendices 1 and 2). Time point is every month following surgical intervention for a total of 6 months. To measure secondary outcomes, a specific cage (see Figure 4) was built with a frontal window and a feeder.

*Figure 4. TEMPOJIMS main facilities.*

Imaging analysis: preoperative CT is performed on all sheep. After animal sacrifice, TMJ blocks are scanned by CT and imaging evaluation is performed using the criteria and score described in Table 1.
Table 1. TEMPOJIMS imaging evaluation criteria.

<table>
<thead>
<tr>
<th>Items</th>
<th>Criteria</th>
<th>0 (no change)</th>
<th>1 (mild change)</th>
<th>2 (moderate change)</th>
<th>3 (severe change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape</td>
<td>Change of joint form</td>
<td>May include reformed joint</td>
<td>Small changes; this change may include ≤2 osteophytes</td>
<td>Moderate changes; multiple osteophytes</td>
<td>Severe changes and new growth; marginal proliferation</td>
</tr>
<tr>
<td>Condyle erosion</td>
<td>Concavity in cortical</td>
<td>This stage includes normal joint with no signs of condyle erosion</td>
<td>Erosion in one-third of joint surface</td>
<td>Erosion in two-thirds of joint surface</td>
<td>Erosion over all joint surface</td>
</tr>
<tr>
<td>Temporal erosion</td>
<td>Concavity in cortical</td>
<td>This stage includes normal joint with no signs of temporal erosion</td>
<td>Erosion in one-third of joint surface</td>
<td>Erosion in two-thirds of joint surface</td>
<td>Erosion over all joint surface</td>
</tr>
<tr>
<td>Condyle sclerosis</td>
<td>Cortical thickening of condyle</td>
<td>This stage includes normal joint with no signs of condyle sclerosis</td>
<td>Sclerosis in one-third of joint surface</td>
<td>Sclerosis in two-thirds of joint surface</td>
<td>Sclerosis over all joint surface</td>
</tr>
<tr>
<td>Temporal sclerosis</td>
<td>Cortical thickening of temporal fossa</td>
<td>This stage includes normal joint with no signs of temporal sclerosis</td>
<td>Sclerosis in one-third of joint surface</td>
<td>Sclerosis in two-thirds of joint surface</td>
<td>Sclerosis over all joint surface</td>
</tr>
<tr>
<td>Condyle marrow</td>
<td>Change of underlying trabecular bone</td>
<td>This stage includes normal joint with no signs of condyle trabecular bone</td>
<td>Sclerosis in less than half of trabecular bone</td>
<td>Sclerosis in half of trabecular bone</td>
<td>Sclerosis in all trabecular bone</td>
</tr>
<tr>
<td>Temporal marrow</td>
<td>Change of underlying trabecular bone</td>
<td>This stage includes normal joint with no change of temporal trabecular bone</td>
<td>Sclerosis in less than half of trabecular bone</td>
<td>Sclerosis in half of trabecular bone</td>
<td>Sclerosis in all trabecular bone</td>
</tr>
<tr>
<td>Calcification</td>
<td>Development of calcification across joint space</td>
<td>No calcification across joint space</td>
<td>Calcification in one-third of joint surface</td>
<td>Calcification in two-thirds of joint surface</td>
<td>Bone fusion across joint space</td>
</tr>
<tr>
<td>Global appreciation</td>
<td>Normal joint</td>
<td>In general, mild changes</td>
<td>In general, moderate changes</td>
<td>In general, severe changes</td>
<td></td>
</tr>
</tbody>
</table>

This assessment is performed and classified independently by two experienced radiologists who will be blinded to intervention. A third radiologist will act as arbiter in case of disparity.

**Absolute masticatory time:** respecting the flowchart (Figure 2), at 9:00 am the animals are placed in individual cages. A dose of 150 grams of dry pellets (Rico Gado A3®) are introduced in the feeder and the time until they eat all the pellets is measured with a chronometer (see Multimedia Appendix 1).

**Ruminant time per cycle:** respecting the timetable (Figure 2), we record 15 ruminatory cycles approximately 4 hours after 150 gram feeding. We use a Canon 7D video camera and images with 25 frames per second. Then, the number of frames per cycle are divided by 25 to obtain time in seconds per cycle (see Multimedia Appendix 2).

**Ruminant kinetics:** we use the software Foundry Nuke (2D tracking) to perform the ruminatory tracking and to obtain the ruminatory cycle average. With the software After Effects, we convert the 2-D tracking into a geometric form (see Multimedia Appendix 2).
**Ruminant area**: we determine the average of 15 cycles and create a geometric form. Using the software Image J, we perform a quantitative measure in pixels of the ruminant area average.

**Weight**: according to the timetable, after eating 150 grams of dry pellets the sheep are weighed (see Multimedia Appendix 1).

All assessments are performed by researchers who are blinded to surgical intervention.

**Statistical Analyses**
All statistical analyses will be performed using the SPSS version 22 (IBM Corp, Armonk, NY, USA). A cross-sectional analysis will be performed to compare the outcome variables in the three levels of the independent variable before and after the randomized treatment group assignment. In the cross-sectional analyses, one-way analysis of variance (ANOVA) will be performed, after testing all the assumptions. For longitudinal analysis, one-way ANOVA with repeated measures will be performed taking as within-subjects effects observations after surgery (months 1 to 6). Fisher least significant difference will be performed as post hoc tests to check for significant differences for the different treatments.

**Reporting of Adverse Events**
Adverse events related to the study will be considered, including (1) anesthesia events: idiopathic death, pneumothorax, other complications related to anesthesia; (2) surgical technique: massive bleeding, condylar fracture, other complications related to surgical technique; and (3) postoperative events: TMJ infection, suture dehiscence, decreased appetite, facial paresis, decreased rumination, decreased weight.

**Discussion**
This study investigates the effects and adverse effects of (1) bilateral discectomy, (2) bilateral discopexy, and (3) bioengineered disk implants. Although this preclinical study will primarily serve as a pilot study, we expect to gain a better understanding
of the morphologic and histologic changes in TMJ and implications in masticatory kinetics. 

So far, results on discectomy are conflicting. Previous preclinical studies in this field [48,53,64,65,68,113–117] have used the contralateral unoperated side as a control and different animal models ranging from mice to a canine model. Using the contralateral side as a control can be inappropriate considering contralateral overload influence. Theoretically, we expect to reduce this bias using a bilateral approach. Animal variability in the different studies is a warning about the importance of using the same animal model in further studies regarding TMJ implant investigations. Therefore, our group performed a previous study considering black Merino sheep as a promisor animal model for studies regarding TMJ disk implants investigation, TMJ prosthesis, and TMJ osteoarthritis model. To increase the quality of TEMPOJIMS the authors will use a sham surgery control group.

We expect to obtain valuable information related to the phase 1 discopexy group regarding if the surgical approach promotes intra-articular damage. This can improve future conclusions about attributing possible damage to the intervention itself instead of the TMJ implant. This question is important considering that a surgical approach to place TMJ implants in phase 2 will be required. Again, using a bilateral intervention could reduce a possible bias. Most preclinical studies have focused on gross morphological/histological assessments and were not designed to characterize the fundamental altered joint movement (kinetics) or functional consequences. In this study, we include pilot secondary outcomes to evaluate changes in ruminant kinetics. We expect to correlate the primary with the secondary outcomes to understand if they can be used in future TMJ studies. It may be interesting to understand several items:

1. Are there differences regarding masticatory time in the disk groups versus discectomy and discopexy?
2. Is there a correlation between histologic and imaging and kinetics results?
3. Does the ruminant area and geometry change when performing different interventions?
4. Is there a difference regarding ruminant kinetics in the disk groups versus discectomy and discopexy?
5. Do TMJ implants accelerate osteoarthritis?
Concerning phase 2, the choice of biomaterial is critical. The TMJ implant will be exposed in a mechanical, stressful environment with a limited blood supply that can limit cell migration and in situ regeneration. Testing three different bioengineering discs in vivo and correlating in vitro with in vivo behavior can seriously improve bioengineering strategies to achieve a safe and efficacious TMJ disk implant for humans. The main strength of this study is the animal model proposed; the conventional and pilot outcomes described; the study design with a randomized, blinded, and placebo control group; and the use of bilateral surgical procedures. Potential limitations of the study include the relatively small sample size. If this study confirms the feasibility of the proposed protocol and initial efficacy of the TMJ disk implants planned, a larger preclinical trial would be warranted to further determine the effectiveness of these discs and promote translation of animal evidence to clinical practice in humans.

**Trial Status**
At the time of submission, the surgical interventions of phase 1 were ongoing at Faculdade de Medicina Veterinária de Lisboa and TEMPOJIMS facilities in Portugal.

**Acknowledgments**
This preclinical trial is supported by Faculdade de Medicina Veterinária da Universidade de Lisboa, Instituto Politécnico de Leiria (Centre for Rapid and Sustainable Product Development), Centro Hospitalar de Setúbal, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa. The authors are grateful to Joaquim Ferreira from Lisbon Faculty of Medicine for study design; to Susan Smith from Institute of Bone and Joint Research-Northern Sydney Local Health District-Sydney Medical School Northern, Australia, for histological analysis; to Pedro Nunes from Radiology Department of Centro Hospitalar Lisboa Norte; to Miguel Virgílio for kinematics video recording; and to Joaquim Ângelo and Ermelinda Ângelo for animal logistics control. This study was granted by Portuguese Grunenthal Foundation and by Secção Regional Oeste da Ordem dos Médicos. This publication was supported by the Portuguese Foundation for Science and
Technology (FCT) through the following projects: UID/Multi/04044/2013 and PTDC/EMS-SIS/7032/2014.

Authors' Contributions
The contributors, with input from the other investigators, conceived this study protocol. JF, RF, NG, AT, NG, and DA developed the protocol and study materials with input from all investigators. NG, AT, and DA participated in the randomization process. LM will conduct the statistical analyses. FM, RG, and SF will participate in the surgical interventions. CB and SC are the coordinators of the veterinary staff and responsible for the animal anesthesia and animal welfare. DC participated in organization support and was study advisor. PM, NA, and MC are dedicated to disk implants 1 and 2. WY, JE, and GJ are dedicated to disk implant 3. SR will coordinate the imaging evaluation. MP and FB are responsible for processing the histologic samples and preparing sections. LC group will coordinate histologic scoring system. All authors read and approved the final manuscript.

Conflicts of Interest
None declared.
CAPÍTULO 6. EFEITO DA DISCECTOMIA E DISCOPEXIA BILATERAL NA CINEMÁTICA MASTIGATÓRIA DA OVELHA BLACK MERINO. ESTUDO PRÉ-CLÍNICO RANDOMIZADO EM OCULTAÇÃO

Trabalho 4

Effects of bilateral discectomy and bilateral discopexy on Black Merino Sheep rumination kinematics: TEMPOJIMS – phase 1 - a randomized preclinical trial

David Faustino Ângelo, Florencio Monje Gil, Raúl González-García, Lisete Mónico, Carla Moura, Luis Carlos Francisco, David Sanz, Nuno Alves, Francisco Salvado, Pedro Morouço

Effects of bilateral discectomy and bilateral discopexy on Black Merino Sheep rumination kinematics: TEMPOJIMS – a randomized preclinical trial

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KEYWORDS
TMJ preclinical trial, TMJ discopexy, TMJ discectomy, TMJ kinematics, TMJ Black Merino Sheep, TMJ animal model
ABSTRACT

Background
The temporomandibular joint interpositional study (TEMPOJIMS) is a rigorous preclinical trial divided in 2 phases. In phase 1 the authors investigated the role of the TMJ disc and in phase 2 the authors evaluated 3 different interpositional materials. The present work of TEMPOJIMS - phase 1, investigated the effects of bilateral discectomy and discopexy in sheep mastication and rumination.

Methods
This randomized, blinded and controlled preclinical trial (in line with the ARRIVE guidelines) was conducted in 9 Black Merino sheep to evaluate changes in mastication and rumination after bilateral discectomy and bilateral discopexy, by comparing with a sham surgery control group. The outcomes evaluated were: (1) absolute masticatory time; (2) ruminant time per cycle; (3) ruminant kinematics, and (4) ruminant area. After baseline evaluation and surgical interventions, the outcomes were recorded over 3 successive days, every 30 days, for 6 months.

Results
The first month after intervention seemed to be the critical period for significant kinematic changes in the discectomy and discopexy groups. However, 6 months after the bilateral interventions, no significant changes were noticed when compared with the control group.

Conclusions
In this study, bilateral discectomy and discopexy had no significant effect in mastication and ruminatory movement. The introduction of kinematic evaluation presents a new challenge that may contribute to the improvement of future studies on the TMJ domain.
INTRODUCTION

The domain of temporomandibular joint (TMJ) bioengineering is growing fast and the potential for obtaining a TMJ interpositional disc is immense. For that purpose, rigorous preclinical trials are needed to progress in translational medicine. However, before using valuable efforts and funds in TMJ bioengineering it is important to clarify knowledge related to the effects induced at the temporomandibular joint by surgical interventions.

TMJ discectomy is the most common intracapsular surgery performed. With overall good results, this technique remains a reasonable choice for internal derangement not responding to nonsurgical treatment [42–46]. Nevertheless, it still is a controversial technique, as it does not restore structural or biological properties of the TMJ [118]. Despite the large number of discectomy procedures performed annually, to the best of our knowledge there are no randomized controlled trials that have investigated, in human or animal, the jaw movement implication of bilateral discectomy and bilateral discopexy, using a sham surgery control group.

Small-size, mid-size and large animal model have been used to investigate the histological effects of unilateral discectomy [47,49,57,114,116], leading to diverse results. It has been stated minor degenerative changes to TMJ ankylosis reports, probably due to limitations regarding animal choice, study design and the use of a unilateral approach with contralateral side as a control, which may have induced bias on available results. As reported in the survey, commissioned by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) [58], only 59% of the 271 randomly chosen articles assessed stated the hypothesis or objective of the study, and the number and characteristics of the animals used (i.e., species/strain, sex, and age/weight). Most of the papers surveyed did not report using randomization (87%) or blinding (86%) to reduce bias in animal selection and outcome assessment. Only 70% of the publications that used statistical methods fully described them and presented the results with a measure of precision or variability [119]. These findings are a cause for concern and are consistent with reviews of many research areas, including clinical studies, published in recent years [58,120,121].
Furthermore, most of the previous studies have focused in the histologic and imaging changes, but additional inputs are mandatory to obtain a clear understanding about the functionality of the TMJ. With this paper the authors will report for the first time a high-quality preclinical study, evaluating the impact of bilateral discectomy and bilateral discopexy, comparing to a sham surgery control group, in mastication and rumination kinematics of Black Merino sheep.

The evaluation of the mastication and rumination kinematics of the sheep jaw were based in the normal processes used by ruminants to breakdown particulate dry matter: (1) initial chewing during eating, and (2) further chewing during rumination [122]. The authors discriminate the two processes and analyzed them separately. To analyze the initial chewing we proposed to examine the time spent to eat a dose of dry pellets. We named this outcome absolute masticatory time. With this outcome we expected to determine if TMJ surgical interventions could induce significant changes for the chewing time. To analyze the ruminant chewing phase a special cage was created and 15 ruminant chewing cycles were recorded with a video camera. Using Foundry Nuke (2D tracking) and Image J software ruminant movements in the frontal plane were analyzed to obtain: (1) ruminant time per cycle, (2) ruminant trajectory and (3) ruminant area. We expected to determine if TMJ surgical interventions could induce significant changes over the rumination movement. Temporomandibular Joint Interpositional Material Study (TEMPOJIMS) was planned with a rigorous design according to the ARRIVE guidelines [58]. An exploratory randomized preclinical study with blinded outcomes preclinical trial was needed in this field to increase the quality of further TMJ studies, to progress in future treatment options for patients undergoing surgery for TMJ disc replacement, and to improve interpretation of future studies regarding TMJ interpositional materials.
MATERIALS AND METHODS

TEMPOJIMS study is a preclinical study divided in 2 phases [123]. This manuscript focus on kinematics outcomes of phase 1, aiming to understand the role of TMJ bilateral discectomy versus TMJ bilateral discopexy, comparing to a sham surgery control group in Black Merino sheep mastication and rumination.

Study Design

The rationale and protocol for the TEMPOJIMS preclinical trial are publicly available [123].

Study Population and Sample

A variety of strains/breeds of sheep have been used in previously TMJ investigation. To decrease biological variability, the authors performed this study in Black Merino sheep strain [109]. In phase 1 the authors used 10 Black Merino sheep with the following inclusion criteria: certified Black Merino sheep, adult (aged between 2 and 5 years [112]), female and in good health condition (veterinary check-up was performed to all animals).

Randomization

The randomization process was performed by a statistical group not involved in the outcome assessments. Ten sheep were randomly allocated to intervention group: bilateral discectomy group (n=3), bilateral discopexy group (n=3), sham surgery group (n=3) and backup group (n=1). One backup sheep was planned to be used if death occurred due to anesthesia or other complication not related to surgical intervention. The allocation to each randomized group was performed preoperatively by sealed envelope (figure 1).
Figure 1. TEMPOJIMS phase 1 enrolment. Baseline assessments evaluated: (1) absolute masticatory time (a dose of 150 grams of dry pellets (Rico Gado A3) were introduced in the feeder and the time until they eat all the pellets were chronometered); (2) ruminant time per cycle (we recorded with a Canon 7D video camera 15 ruminant cycles approximately four hours after 150 gr feeding; (3) ruminant kinematics and (4) ruminant area (we used software Foundry Nuke (2D tracking) to make the jaw tracking and to have a With the software After Effects, we converted the 2D tracking in a geometric form).

**Procedures**

Ten eligible sheep were assigned to their baseline pilot secondary outcomes measured at days 11, 10, and 9 before surgery in central TEMPOJIMS facilities (figure 2). Transportation to surgical facilities was performed 5 days before surgery to avoid animal stress and allow familiarization to temporary accommodations. The surgical team was not blinded to treatment allocation given the type of intervention; however surgical team members were not involved in outcome assessment. Serious adverse events were defined as events that were fatal or life-threatening or persistent disability, which resulted in death, more than 10% weight loss per week, or clinically significant hazard or harm to the animal.
Figure 2. Flow Chart of TEMPOJIMS phase 1.

Anesthesia Protocol

Fasting and water restriction were required 24 hours before surgery. Sedation was performed with diazepam (0.5 mg / kg i.v.), followed by anesthesia induction with ketamine (5 mg / kg i.v.). Oral intubation was performed and anesthesia was maintained with isoflurane (1.5 to 2%). To assure animal analgesia, Meloxicam (0.5 mg / kg i.v., bid) was administered in surgery day and during 4 days post-operatively. Antibiotic prophylaxis with amoxicillin and clavulanic acid were administrated for 5 days.

Surgical Intervention

(A) Bilateral discectomy (n = 3): under general anesthesia, the surgical team performed a preauricular incision and a blunt dissection of the soft tissue covering the joint. The joint area was disclosed and the articular capsule was incised. The disc and its attachments were identified. The medial, anterior, posterior and lateral disc attachments were detached and discectomy was performed. The wound was closed in layers with Vicryl 3/0.

(B) Bilateral discopexy (n = 3): under general anesthesia, the surgical team performed a preauricular incision and a blunt dissection of the soft tissue covering
the joint. The joint area was disclosed and the articular capsule was incised. The
disc and its attachments were identified. The lateral and posterior disc attachments
were detached and sutured with PDS 3/0. The wound was closed in layers with
Vicryl 3/0.

(C) Sham surgery (n = 3): under general anesthesia, the surgical team performed
a preauricular incision and a blunt dissection of the soft tissue covering the joint.
The TMJ articular capsule was not incised. The wound was closed in layers with
Vicryl 3/0.

Follow-up assessments

Baseline assessment (T0) were performed before surgery in day -11,-10,-9 (table
1). Ten days after surgery, animals were transported to TEMPOJIMS facilities. Day
19, 20 and 21 (T1) after surgery the follow-up pilot outcomes started to be recorded:
every 30 days for 6 months (figure 2). T0-T6 was based on a mean of the 3
measurements. The assessments were performed by 2 specially trained assessors
who were not affiliated with the intervention. All animals had bilateral scar to reduce
possible bias.

Kinematic outcomes

Kinematic outcomes evaluated were: (1) absolute masticatory time; (2) ruminant
time per cycle; (3) ruminant trajectory; (4) ruminant area.
To measure the referred outcomes, a specific cage was built with a frontal window
and a feeder. All assessments were performed by researchers’ blinded to surgical
intervention, and were designed to evaluate masticatory changes related to
masticatory time and to ruminant kinematics. These outcomes included:

(1) Absolute masticatory time: respecting timetable (figure 2), at 9:00 am the 10
sheep were placed in the individual cage. A dose of 150 grams of dry pellets
(Rico Gado A3®) were introduced in the feeder and the time until they eat all the
pellets was measured with a chronometer;
(2) Ruminant time per cycle: respecting timetable (figure 2), we recorded 15 ruminant cycles approximately 4 hours after 150 grams feeding. We used a Canon 7D Video Camera and images were recorded with 25 frames per second. Then, the number of frames per cycle was divided by 25 to obtain time in seconds per cycle;

(3) Ruminant trajectory: we used the software Foundry Nuke (2D tracking) to make the jaw tracking and calculate the ruminant cycle average. With the software After Effects, we converted the 2D tracking in a geometric form (figure 3);

(4) Ruminant area: we determined the average of 15 cycles, and created a geometric form. Using the software Image J, we performed a quantitative measure in pixels of the ruminant area average (figure 3).

Figure 3. Ruminant cycle kinematics (A) – initial position; (B) - maximum open mouth; (C) - maximum lateral movement; (D) – end of ruminant cycle.
Statistical analysis

TEMPOJIMS phase 1 pilot randomized controlled preclinical trial used 9 Black Merino Sheep with a 6 months follow-up. Our primary analysis tested the effects of the independent variable (IV) of 3 experimental conditions: 1 = bilateral discectomy; 2 = bilateral discopexy; 3 = sham surgery, using series pre-test (T0) and post-test (T1 to T6). As dependent variables (outcome measures) we measured: the time to eat 150gr of pellets, the ruminant time per cycle and the ruminant area. These events were measured 3 times in the pre-test for supporting invariance concerning the outcome measures before the surgical intervention (IV). Our secondary tests (post-test) analyzed the outcomes measuring 3 times, in 6 time-points, one per month at the same place, date and hour as in pre-test (figure 2).

All statistical analyses were performed using the Statistical Package for Social Sciences (IBM SPSS, version 22.0). Shapiro-Wilk tests were performed in pre-test (T0) and post-test (T1 to T6), showing a normal distribution in all groups per time (p > .0.05), except for T4 and T6 for discopexy in ruminant area (Shapiro-Wilk = .761 and .384, p < .05). Other outcomes showed a normal distribution (p > .05). Additionally, Levene Statistics were performed for testing the homogeneity of variances and statistical significant results were found for T1, T2 and T5 in ruminant area (Levene statistics = 8.59, 6.35, and 7.82, p < .05), which lead to calculated non-parametric tests for these moments. For pre-test and the other times groups’ variances were homogeneous (p > .07), which lead us to perform parametric tests. A one-way Analysis of Variance (ANOVA) (or the non-parametric equivalent Kruskal-Wallis test) was performed for cross-sectional analysis, in order to compare the outcome variables in the three levels of the IV before and after the random treatment group assignment. Fisher LSD and Games-Howell Post-hoc tests were performed for equal variances assumed and not assumed, respectively. For longitudinal analysis, Mauchly’s test of sphericity was non-significant for absolute masticatory time (Mauchly’s W = .004, p = .589), allowing to perform a parametric test one-way ANOVA with repeated measures, taking as within-subjects effects observations before (T0) and after surgery (T1 to T6) for bilateral discectomy, bilateral discopexy, and sham surgery conditions. For ruminant area a Greenhouse-Geisser corrected test was used, due to Mauchly’s W = .000, p = .011.
RESULTS

Descriptive baseline statistics are presented in table 1. A total of 4 outcomes were analyzed: (1) absolute masticatory time; (2) ruminant time per cycle; (3) ruminant trajectory and (4) ruminant area.

<table>
<thead>
<tr>
<th>Sheep ID</th>
<th>Age</th>
<th>Birth-date</th>
<th>Weight §</th>
<th>Absolute Masticatory Time ψ</th>
<th>Ruminant kinetics and Area †</th>
<th>Ruminant Time per cycle ‡</th>
<th>Allocation</th>
</tr>
</thead>
<tbody>
<tr>
<td>8298</td>
<td>11.01.11</td>
<td>56.0 Kg</td>
<td>85.0</td>
<td>6449</td>
<td>0.74 sec</td>
<td>Discopexy</td>
<td></td>
</tr>
<tr>
<td>9705</td>
<td>02.04.12</td>
<td>70.3 Kg</td>
<td>90.7</td>
<td>5252</td>
<td>1.21 sec</td>
<td>Discectomy</td>
<td></td>
</tr>
<tr>
<td>8264</td>
<td>19.07.10</td>
<td>56.3 Kg</td>
<td>79.3</td>
<td>7223</td>
<td>0.87 sec</td>
<td>Sham</td>
<td></td>
</tr>
<tr>
<td>9982</td>
<td>02.09.12</td>
<td>56.0 Kg</td>
<td>76.0</td>
<td>6591</td>
<td>0.94 sec</td>
<td>Discopexy</td>
<td></td>
</tr>
<tr>
<td>3969</td>
<td>30.10.09</td>
<td>57.0 Kg</td>
<td>89.7</td>
<td>7768</td>
<td>0.90 sec</td>
<td>Sham</td>
<td></td>
</tr>
<tr>
<td>8284</td>
<td>16.02.11</td>
<td>68.0 Kg</td>
<td>97.7</td>
<td>6904</td>
<td>0.93 sec</td>
<td>Discopexy</td>
<td></td>
</tr>
<tr>
<td>8267</td>
<td>13.07.10</td>
<td>75.7 Kg</td>
<td>71.7</td>
<td>8846</td>
<td>0.73 sec</td>
<td>Discectomy</td>
<td></td>
</tr>
<tr>
<td>9701</td>
<td>07.04.12</td>
<td>63.0 Kg</td>
<td>108.7</td>
<td>10354</td>
<td>1.14 sec</td>
<td>Discopexy</td>
<td></td>
</tr>
<tr>
<td>1903</td>
<td>25.12.12</td>
<td>52.0 Kg</td>
<td>101.3</td>
<td>6007</td>
<td>0.74 sec</td>
<td>Sham</td>
<td></td>
</tr>
</tbody>
</table>

ϕ No significant differences between sheep in the reported characteristics were found at baseline, p > .10.

§ Weight is presented in kilograms.

ψ The absolute masticatory time is measured at 9:00 am when a dose of 150 grams of dry pellets (Rico Gado A3®) is introduced in the feeder and the time until they eat all the pellets is chronometered in seconds.

† The ruminant kinetics is the average tracking of 15 ruminant cycles and created a geometric form using the software Image J. A quantitative measure in pixels of the ruminant area average is presented.

‡ Respecting the time per cycle we recorded 15 ruminant cycles approximately four hours after 150 gr feeding. We use a Canon 7D Video Camera and images with 25 frames per second. Then, the number of frames per cycle was divided by 25 to obtain time in seconds per cycle.

| Table 1. Baseline descriptive statistics |
(1) ABSOLUTE MASTICATORY TIME

Cross-sectional analysis. We compared the absolute masticatory time of the 3 groups each month post-surgery (T1 to T6). A one-way ANOVA (or the non-parametric equivalent Kruskal-Wallis test) was performed, showing significant differences between the 3 groups only in T1, \( p = .03 \) (one-tailed), effect size of \( \eta^2_p = .736, (1 – \beta) = .804 \) (table 2), due to the higher values in the discopexy in comparison with sham surgery, as Games-Howell post-hoc test showed, \( p = .028 \). Throughout the baseline and the remaining follow-up period (T2-T6), no statistically differences were found between discectomy, discopexy, and sham surgery conditions (\( p > .20 \)).

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discectomy</td>
<td>79.4±10.0</td>
<td>102.1±6.5</td>
<td>86.0±17.6</td>
<td>79.3±13.2</td>
<td>85.2±11.9</td>
<td>78.3±7.2</td>
<td>74.7±5.0</td>
</tr>
<tr>
<td>Discopexy</td>
<td>97.1±11.8</td>
<td>108.2±5.4</td>
<td>90.7±9.2</td>
<td>95.4±15.7</td>
<td>98.2±18.0</td>
<td>95.0±12.1</td>
<td>92.6±7.6</td>
</tr>
<tr>
<td>Sham</td>
<td>90.1±11.0</td>
<td>89.2±5.5</td>
<td>80.3±10.8</td>
<td>84.7±16.2</td>
<td>94.9±13.7</td>
<td>82.6±11.7</td>
<td>85.6±18.2</td>
</tr>
</tbody>
</table>

\[
\begin{array}{c}
F_{(2,5)}^* \\
K-W X^2(2) \\
\eta^2_p \\
1 – \beta
\end{array}
\begin{array}{c}
1.98 \\
5.54^* \\
.397 \\
.27
\end{array}
\begin{array}{c}
.89 \\
.80 \\
.74 \\
.80
\end{array}
\begin{array}{c}
.63 \\
.14 \\
.10 \\
.10
\end{array}
\begin{array}{c}
2.02 \\
.23 \\
.14 \\
.14
\end{array}
\begin{array}{c}
1.76 \\
.17 \\
.11 \\
.11
\end{array}
\begin{array}{c}
.80 \\
.40 \\
.40 \\
.40
\end{array}
\begin{array}{c}
.27 \\
.37 \\
.27 \\
.24
\end{array}
\]

\( p = .03 \) one-tailed test

**Table 2.** Absolute masticatory time for T0 (baseline) to T1 to T6 (post-test): Descriptive, one-way ANOVA for T0 and T3 to T6 and Kruskal-Wallis for T1 and T2, effect-sizes (\( \eta^2_p \)) and observed power (\( 1 – \beta \))

Longitudinal analysis. A one-way ANOVA with repeated measures was performed, taking as within-subjects effects months before (T0) and after surgery (T1 to T6) for discectomy, discopexy, and sham surgery conditions. Significant effects across time were found for discectomy, \( F(6, 12) = 5.67, p = .005, \eta^2_p = .739, (1 – \beta) = .947 \), but not for discopexy and sham surgery, \( F(6, 12) = 2.65 \) and \( 1.59, p > .07, \eta^2_p = .570 \) and \( .443, (1 – \beta) = .635 \) and \( .403 \), respectively.
Effects of bilateral discectomy and bilateral discopexy on Black Merino Sheep rumination kinematics: TEMPOJIMS – phase 1 - a randomized preclinical trial

Table 3. Comparison of absolute masticatory between baseline and months 1 to 6: within-subjects contrasts, effect-sizes ($\eta^2_p$), and observed power ($1 - \beta$)

Considering the differences in relation to the baseline (table 3), the within-subjects contrasts identified only an increase statistically significant for discectomy between T0 and T1 (effect size of 90%; observed power of .60) and between T0 and T4 (effect size of 93%; observed power of .74). For discopexy and sham surgery, despite the effect sizes, considering the low observed powers, the differences in relation to the baseline were not statistically significant. Figure 4 represents absolute masticatory time in the baseline and from T1 to T6.

Figure 4. Absolute masticatory time from T0 (baseline) to T1 to T6 (post-test) in discectomy, discopexy, and sham surgery conditions.
(2) RUMINANT TIME PER CYCLE

Cross-sectional analysis. Ruminant time per cycle rate did not vary across groups both in the pre-test (T0) and in all times for the post-test (p > .20), as shown in table 4.

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discetomy</td>
<td>.93±.03</td>
<td>.79±.01</td>
<td>.91±.06</td>
<td>.69±.03</td>
<td>.89±.09</td>
<td>.98±.20</td>
<td>1.01±.18</td>
</tr>
<tr>
<td>Discopexy</td>
<td>.69±.10</td>
<td>.76±.11</td>
<td>.83±.12</td>
<td>.62±.09</td>
<td>.89±.05</td>
<td>.91±.06</td>
<td>.98±.15</td>
</tr>
<tr>
<td>Sham</td>
<td>.80±.13</td>
<td>.72±.12</td>
<td>.85±.17</td>
<td>.66±.07</td>
<td>.76±.11</td>
<td>.88±.21</td>
<td>.84±.08</td>
</tr>
</tbody>
</table>

\[
F_{(2,5)}^{*} = 3.02, \quad \eta^2_p = .547, \quad 1 - \beta = .351
\]

no significant effects, p > .05

Table 4. Ruminant time per cycle rate for T0 to T1-T6: descriptive, one-way ANOVA, effect-sizes (\(\eta^2_p\)) and observed power (1 – \(\beta\))

Longitudinal analysis. A one-way ANOVA with repeated measures was performed, taking as within-subjects effects the baseline and the six months after surgery for discectomy, discopexy, and sham surgery. A significant effect across time was found for discopexy and sham, respectively, \(F(6, 6) = 6.87\) and \(4.11, p < .018, \eta^2_p = .773\) and \(.673, (1 – \beta) = .977\) and \(.845\), but not for discectomy, \(F(6, 6) = 2.70, p = .126, \eta^2_p = .730, (1 – \beta) = .455\).

The comparison of ruminant time per cycle rate between baseline and months after the surgery identified two differences for discopexy, however only one (T5 vs. T0) with an acceptable power (effect size of 95%). For discectomy and sham surgery no significant differences were found in relation to baseline. Figure 5 illustrates the ruminant time per cycle rate in the baseline and from T1 to T6. As can be seen, lower scores were obtained in T1, T2 and T3, seeming that sheep start recovering in times T4, T5, and T6.
Figure 5. Ruminant time per cycle rate for T0 (pre-test) to T1 to T6 (post-test) in discectomy, discopexy, and sham surgery.

(3) RUMINANT TRAJECTORY AND AREA

Descriptive results of ruminant trajectory and average area of ruminination are presented in figure 6.

Figure 6. Ruminant kinematics and average area of ruminination for the nine sheep.* no ruminant cycles were detected - ruminant area assumed value as zero.
Cross-sectional analysis. Masticatory areas only varied across groups in T3 and T4. For T3 the Fisher LSD post-hoc test identified a significant superiority in the discopexy area in comparison with the discectomy area, \( p = .008 \). Longitudinal analysis. A one-way ANOVA with repeated measures with Greenhouse-Geisser corrected, taking as within-subjects effects the baseline and the six months after surgery (T1 to T6) for discectomy, discopexy, and sham surgery, did not show statistically significant differences for the three conditions (\( p > .10 \)). The differences from pre to post-test times were also no statistically significant (\( p > .05 \), with small power, since \((1 - \beta) < .80\), as can be seen in table 7.

<table>
<thead>
<tr>
<th>Comparison with baseline (T0)</th>
<th>Discectomy</th>
<th>Discopexy</th>
<th>Sham Surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( F )</td>
<td>( \eta^2_p )</td>
<td>( 1 - \beta )</td>
</tr>
<tr>
<td>T1 vs. T0</td>
<td>0.29</td>
<td>.13</td>
<td>.06</td>
</tr>
<tr>
<td>T2 vs. T0</td>
<td>0.42</td>
<td>.17</td>
<td>.07</td>
</tr>
<tr>
<td>T3 vs. T0</td>
<td>2.37</td>
<td>.54</td>
<td>.15</td>
</tr>
<tr>
<td>T4 vs. T0</td>
<td>0.25</td>
<td>.11</td>
<td>.06</td>
</tr>
<tr>
<td>T5 vs. T0</td>
<td>0.00</td>
<td>.00</td>
<td>.05</td>
</tr>
<tr>
<td>T6 vs. T0</td>
<td>0.61</td>
<td>.23</td>
<td>.08</td>
</tr>
</tbody>
</table>

Table 7. Comparison of ruminant area between baseline and T1 to T6: within-subjects contrasts, effect-sizes (\( \eta^2_p \)), and observed power (1 − β).

Figure 7 represents ruminant area for T0 (pre-test) to T1 to T6 (post-test) in discectomy, discopexy, and sham surgery. As can be seen, the baseline (pre-test) are similar for the three experimental conditions. After surgery ruminant areas are lower in discectomy condition, although the differences were not statistically significant.
ADVERSE EVENTS
No serious adverse events were reported apart of one sheep from discectomy group that stopped rumination in T1 and T2, returning to normal function in T3 to T6.

DISCUSSION
The main goal of the present work was to examine possible effects on the sheep kinematics of mastication and rumination, induced by different types of surgery. The proposed methodology has proven to be feasible and sensitive to the interventions. Homogenous conditions were obtained in baseline, the animals behaved properly in front of the camera, guaranteeing the quality of the kinematic assessments (figure 3).

The measurement of kinematics was intended to progress in the understanding of TMJ surgery implications for the jaw movements. Theoretically, the introduction of a surgery intervention may induce relevant movement changes [20], which should be quantified to infer about the outcomes. That is why this pioneer study is relevant to establish that, independently of the procedure, sheep have the ability to adapt to survive. Regarding the absolute masticatory time, it
was expected that after bilateral discectomy the animals would increase the time to eat the 150gr of pellets [124]. Accordingly, the discectomy group significantly increased time by 28% in T1. This could be related to pain in TMJ leading to a slower food intake. However, with time, these animals were able to perform a slow progression to baseline values and at the end of the study, the discectomy group was taking similar time than in baseline data (74.67 seconds) (figure 4). As above-mentioned there is a lack of studies comparing the functionality of TMJ according to interventions. Thus, it was not possible to compare this masticatory outcome measure with other investigations. Although there were no statistical differences between the time before and after surgery (ie T0 vs T1), it can be noticed a tendency to take longer time for the ingestion of the 150gr. Progressively this time started decreasing getting similar to the one performed before interventions (T0 vs T6). This outcomes suggest that sheep presented the ability to adapt to the induced constrains, highlighting the importance that function has over form [125]. The authors agree that it would be interesting in the future to analyze this outcome for a longer period. Regarding the ruminant time per cycle attractive results were achieved. In discectomy group was observed that in T1 and T2 one animal stopped rumination. This could alert for the importance of future investigations in this field, where discectomy could have a more important effect in rumination than mastication. The authors believed at first that an ankylosis process could start after bilateral discectomy, but at T3 all animals were ruminating. This output suggests that besides initial slow down related to food intake, rumination area and even one animal without the ability to ruminate, the animal were able to readapt and return to normal particulate breakdown. When analyzing figure 5 it is noticeable that all groups reduced the ruminant time per cycle in T3, without knowing the cause of any event leading to that result. However, in T4-T6 they reassumed expected values. The animals from discectomy and discopexy groups in T5 and T6 demanded more time to achieve a ruminatory cycle, suggesting a less effective rumination process. Examining the area, it is possible to notice that a faster ruminant cycle is obtained through a smaller ruminant area of each cycle. Moreover, one interesting detail is that in T3 and T4 a normalization of the results was observed for the discectomy group. This output
raises the question if 3-4 months after TMJ surgical intervention is the necessary period for remodelation and adaptation?

The evaluation of trajectory and area of rumination was interesting because it was possible to identify a pattern for rumination. Each animal showed a favorite side for rumination but they switched side independently from the intervention. In every animal was noticed a triangular shape trajectory, with high resemblances with the jaw movement in the anesthetized rabbit [126]. After surgery ruminant areas became lower for the discectomy group, although the differences were not statistically significant. In conclusion, present results proved that kinematic jaw analysis is feasible in Black Merino sheep, and that the sham surgery control group was efficacious along time. Further research should be able to examine possible associations between these results with more consensual histologic, imaging and weight outcomes (e.g.[127]). We are not aware of any previous randomized, blinded, preclinical study respecting ARRIVE guidelines in TMJ domain. Using Black Merino sheep, with age and gender selection, protocol publicly available, sham control group and bilateral approach, became critical to reduce possible bias on results. In baseline, the proposed pilot outcomes were homogenous and the sham control group performed effectively in this study. Furthermore, for the present study, performed interventions were bilateral in all groups. Not only, it may clarify a better understanding of TMJ disc role and to reduce possible bias on results, but also it may avoid an adverse effect of the contralateral unoperated joint as reported when unilateral procedures are performed [47]. The first month after intervention seems to be the critical period regarding kinematic changes, with modifications related to absolute masticatory time, ruminant time per cycle and ruminant area, both in discectomy and discopexy groups. Still, after one month, TMJ bilateral discopexy does not seem to have an important impact concerning kinematic changes in Black Merino sheep. On the other hand, TMJ bilateral discectomy seems to have a significant impact, mostly in T1 and T2 but in T3 to T6 a normalization of the results can be observed. The authors agree that the rigorous study design, the animal model and bilateral intervention were the main advantages of this research. The limitations were mostly due to the small sample size, so further
research should aim higher samples. The introduction of kinematics evaluation can introduce a new challenge for future studies in TMJ domain, highlighting the functionality of this critical joint.

Acknowledgements
We would like to consider the valuable work of Miguel Virgílio in the ruminant analysis.

Declarations
Funding: Portuguese Grant for Young Researcher in the pain – Grüenthal

Competing Interests: None declared

Ethical Approval: Portuguese National Authority for Animal Health registered with number 026618

Patient Consent: Not required
CAPÍTULO 7. ANÁLISE HISTOLÓGICA, IMAGIOLÓGICA E DE MASSA CORPORAL APÓS DISCECTOMIA E DISCOPEXIA BILATERAL NUM ESTUDO PRÉ-CLÍNICO RANDOMIZADO EM OCULTAÇÃO

Trabalho 5

A Pilot Preclinical Randomized Controlled Trial of Bilateral Discectomy versus Bilateral Discopexy in Black Merino Sheep Temporomandibular Joint: TEMPOJIMS Phase 1 - Histologic, Imaging and Body Weight results

David Faustino Angelo, Florencio Monje, Raúl González-García, Pedro Morouço, Rita Sousa, Lia Neto, Inês Caldeira, Margaret M. Smith, Susan M. Smith; David Sanz, Fábio Carvalho; Belmira Carrapiço, Sandra Cavaco, Mário Pinho, Carla Moura, Nuno Alves, Lisete Mónico, Francisco Salvado, Christopher B. Little

A Pilot Preclinical Randomized Controlled Trial of Bilateral Discectomy versus Bilateral Discopexy in Black Merino Sheep Temporomandibular Joint: TEMPOJIMS Phase 1 - Histologic, Imaging and Body Weight results

David Faustino Angelo¹,³, Florencio Monje², Raúl González-García², Pedro Morouço³, Rita Sousa⁴, Lia Neto⁴, Inês Caldeira⁴, Margaret M. Smith⁵, Susan M. Smith⁵; David Sanz⁶, Fábio Santos⁷; Belmira Carrapiço⁷, Sandra Cavaco⁸, Mário Pinho⁷, Carla Moura³, Nuno Alves³, Lisete Mónico⁶, Francisco Salvado¹, Christopher B. Little⁵

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KEYWORDS
TMJ cartilage; TMJ discectomy; TMJ discopexy; TMJ preclinical study; TMJ randomized study; Temporomandibular Osteoarthritis;
ABSTRACT

OBJECTIVE: Evaluate histopathologic and imaging impact of bilateral discectomy or discopexy in Black Merino sheep temporomandibular joints (TMJ), using a high-quality randomized, blinded, control trial following the ARRIVE guidelines.

DESIGN: TEMPOJIMS phase 1 is pilot randomized preclinical study, conducted in 9 Black Merino sheep designed to investigate imaging, histopathologic and body weight changes after bilateral TMJ discectomy, discopexy and sham surgery.

RESULTS: Significant changes were noticed in discectomy group, both in imaging and histopathologic analyses. Body weight changes were most pronounced in discectomy group in the first 4 months after surgery with recovery to baseline weight 6 months after surgery. Discopexy induced nonsignificant changes in both imaging and histologic scoring analyses.

CONCLUSION: This study reinforces the importance of developing an effective interpositional material to substitute for the disc and the need to explore the molecular mechanisms that underlie TMJ cartilage degeneration. The study design proposed in TEMPOJIMS study represents important progress towards future rigorous TMJ investigations.
INTRODUCTION

In severe temporomandibular disorders (TMD) the standard treatment is mostly surgical [36]. However, the role of temporomandibular joint (TMJ) surgery is not well defined [128] due to a lack of quality randomized controlled clinical trials comparing TMJ surgical treatment with medical treatment and placebo [129,130]. TMJ open surgical approaches for severe disorders include mostly discectomy, discopexy and, in cases where nothing in the joint is salvable, a total joint replacement may be necessary [36]. Despite the large number of discectomy procedures performed annually, we are not aware of any rigourously-performed, randomized, controlled trials that have investigated in human or animal the effectiveness of discectomy, as compared with discopexy, bioengineered interpositional material and sham surgical interventions. In 1995, Trumpy IG et al, compared three different TMJ surgical treatments: discopexy, discectomy without replacement and discectomy with replacement of the disc with an interpositional implant. They concluded the interpositional implant clearly accelerated TMJ osteoarthrosis (OA). Discectomy and implant groups developed TMJ OA in 93% and 100%, but only 62% in the discoplasty group had OA. However, TMJ discopexy was associated with frequent relapse, requiring secondary discectomy [44]. This study suggests we should improve knowledge in the role of surgery and progress for future interpositional materials.

Most clinical trials use imaging to classify the TMJ degenerative process [42]. Computed tomography (CT) is a valuable tool to evaluate TMJ OA [131] and most clinical studies evaluate articular changes by CT [132–138]. Two important long-term follow-up clinical studies presented condylar flattening and sclerosis after discectomy but did not associate them with TMJ symptoms [134,137]. The Deprez group (1962) suggested an association of articular erosion with pain in the post-operative period [133]. However, while imaging modalities are key measures in clinical research, preclinical studies provide a unique chance to also obtain histologic pathology in order to better understand TMJ surgery-induced changes and improve knowledge in interpositional materials research. Previous preclinical studies have evaluated histologic and imaging outcomes using study designs with a significant potential sources of bias (selection bias, measurement bias, non-
randomization, non-blinded outcome assessment) increasing risk of errors in the results of the study and in conclusions [67,139–143].

The Temporomandibular Joint Interposal Material Study (TEMPOJIMS) was planned with a rigorous pre-published design [123] according to the ARRIVE guidelines [58]. This first high-quality randomized preclinical study, performed in Black Merino sheep, is required to increase the translational power of further studies and to progress future treatment options for patients undergoing surgery for TMJ disc replacement. TEMPOJIMS is divided into phase 1 and 2. Phase 1 is a randomized, blinded preclinical trial designed to investigate the TMJ imaging (CT), histologic, and body weight changes in sheep after bilateral discectomy (n=3), discopexy (n=3) or sham surgery (n=3). Phase 2 uses the same design to test 3 different bioengineering discs to substitute for the disc in sheep TMJ. All the assessments were performed and classified independently by two professionals from each area who were blinded to intervention, and in both phases the primary outcome is the histological grading of TMJ pathology. In this manuscript we report the phase 1 outcomes.

MATERIAL AND METHODS

Study design

This study was conducted predominantly in Portugal, but a number institutions were involved: 1) Lisbon Faculty of Medicine for study design, coordination and statistical analysis; 2) Histology department of Centro Hospitalar de Setúbal for histological preparation; 3) Interdisciplinary Centre of Research in Animal Health in Faculty of Veterinary for veterinary support; 4) Department of Oral and Maxillofacial-Head and Neck Surgery, University Hospital Infanta Cristina, Badajoz, Spain for surgical support; 5) Radiology Department of Santa Maria Hospital, Lisbon, Portugal for imaging analysis; 6) Institute of Bone and Joint Research-Northern Sydney Local Health District-Sydney Medical School Northern, University of Sydney, Australia, for histological analysis; and 7) Coimbra University for statistical analysis. The rationale and protocol for the TEMPOJIMS preclinical trial are publicly available [123]. An
independent data and safety monitoring board unblinded preclinical results. The study was approved by the Portuguese National Authority for Animal Health registered with number 026618. The study design and organization respected the ARRIVE guidelines [58].

Study population and sample

Relevant preclinical TMJ studies have been conducted in sheep [85,88,139,144,145], and to decrease biological variability in TEMPOJIMS results, a specific purebred Black Merino sheep strain was used. In 2016, our group performed an anatomic, biomechanical and histologic study of Black Merino sheep TMJ highlighting the potential of this animal to conduct preclinical trials in the TMJ domain [109]. The following eligibility criteria were used: certified Black Merino sheep, adult (aged between 2 and 5 years [112]), female, and in good health condition (veterinary evaluation was performed in all animals, including ensuring normal dentition (with 32 teeth, 8 mandibular incisors 12 molars and 12 premolars).

Randomization

The randomization process was performed by a statistical group not involved in the outcome assessments. Ten sheep were randomly allocated to intervention group: bilateral sham surgery (n=3), bilateral discectomy (n=3), bilateral discopexy (n=3), and backup group (n=1). One backup sheep was planned to be used if death occurred due to anaesthesia or other complication not related to surgical intervention. The allocation to each randomized group was performed preoperatively by sealed envelope (Figure 1).
Procedures

Ten eligible sheep were assigned to baseline body weight measured at days 11, 10, and 9 before surgery in TEMPOJIMS facilities (Figure 3). Transportation to surgical facilities was performed 5 days before surgery to avoid animal stress and allow familiarization to the temporary accommodation. Head CT-scan was performed on the day of surgery taking advantage of pre-anaesthesia sedation (Figure 2). The surgical team was not blinded to treatment allocation given the type of intervention; however surgical team members were not involved in outcome assessment. Serious adverse events were defined as events that were fatal or life-threatening or persistent disability, that resulted in death, > 10% weight loss per week, or clinically significant hazard or harm to the animal.
Figure 2. Preoperative CT performed under pre-anesthesia sedation in Lisbon Veterinary Faculty.

Intervention phase

Anaesthesia Protocol

Fasting and water restriction were required 24 hours before surgery. Sedation was performed with diazepam (0.5 mg / kg i.v.), followed by anaesthesia induction with ketamine (5 mg / kg i.v.). Oral intubation was performed and anaesthesia was maintained with isoflurane (1.5 to 2%). To assure animal analgesia, meloxicam (0.5 mg / kg i.v., bid) was administered on the day of surgery for 4 days post-operatively. Antibiotic prophylaxis with amoxicillin and clavulanic acid (50mg / kg i.v. bid) were used for 5 days.

Surgical Intervention

In all animals the surgical site was shaved, the skin prepared with povidone iodine solution, and isolated with sterile drapes according to standard surgical procedures. With a 15-scalpel blade we performed a 6cm long pre-auricular skin incision followed by blunt dissection of the soft tissue covering the joint to expose the
The articular capsule and tissue retractors used to maintain exposure of the surgical field. In the Sham Surgery GROUP (n=3) the TMJ articular capsule was not incised, and the wound was closed in 3 layers (muscular, subcutaneous and skin) with Vicryl 3/0. In the remaining animals the joint capsule was incised and the disc and its attachments were identified. In GROUP A - Discectomy (n=3), the disc was exposed, and using iris scissors the lateral, anterior and posterior attachments were dissected allowing exposure and transection of the medial attachment and removal of the disc intact. In GROUP B - Discopexy (n=3) the lateral and posterior disc attachments were sharply detached using an iris scissor. A 4 mm triangular segment of the retrodiscal tissue was removed and then sutured with PDS 3/0. The wound, including joint capsule was closed in 4 layers (joint capsule, muscular, subcutaneous and skin) with Vicryl 3/0.

Follow-up assessments

Ten days after surgery, animals were transported to TEMPOJIMS facilities. Day 19, 20 and 21 (T1) after surgery the follow-up secondary outcomes were recorded, and repeated every 30 days for 6 months (T1 to T6, respectively; Figure 3). T0-T6 was based on a mean of the 3 day measurements of each month. Immediately after euthanasia all animals had a second CT scan and the TMJ block was removed to histology.

![Figure 3. Flow chart of TEMPOJIMS phase 1.](image-url)
Outcomes

**Histologic analysis:** Following euthanasia (six months after surgery) the TMJ was removed intact using a necropsy bone oscillatory saw according to the following anatomic references: cranial - cranial aspect of coronoid process in the union region of the zygomatic process; caudal - external to acoustic meatus; dorsal - the squamous temporal bone; and ventral - 2 cm below the acoustic meatus in the zone of stylohyoid angle. The joints were fixed in 10% buffered formalin for 24 hours and stored in 70% ethanol. Decalcification was obtained by immersion in 10% formic acid in 5% formalin for 20 days with solution changed every 2 days, after which the articulations were cut sagittally through the whole condyle. TMJ articulations were then immersed in three graded methyl salicylate/paraffin mixtures, embedded in paraffin and sectioned through to the central part of TMJ. Four micron sections were mounted on glass slides, heated for 1 hour at 65 degrees, de-waxed with 3 cycles of 5 minutes with xylene, and stained with toluidine blue and fast green as previously described [111]. Slides identified by a number code were randomized and shipped to the Raymond Purves Labs for scoring by 2 blinded independent assessors experienced in evaluating sheep joint histopathology (CBL, MMS).

As the normal histomorphology of the TMJ is quite distinct from cartilage in appendicular synovial joints [146] (Figure 4Aa), a modification of a published scoring system specific for the TMJ was used [56] (Table 1). Briefly, the mandibular and temporal cartilage (structure, cell number, shape and cloning, and proteoglycan content and distribution), tidemark, cement line, and subchondral bone (structure, osteocyte number, osteoblast activation, vascular invasion, and calcified cartilage islands) were separately scored from 0 (normal) to 3 (severe change, >70% abnormal). Additionally, the temporal and retrodiscal synovial hyperplasia, fibrosis and inflammatory cell infiltration were also scored from 0-3. The summed cartilage (possible maximum score 60 in each condyle), subchondral bone (possible maximum score 15 in each condyle), synovial (possible maximum score 9 in each site), and total (possible maximum score 168) histopathology scores were calculated.
<table>
<thead>
<tr>
<th>Site</th>
<th>Structure scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mandibular surface</td>
<td>AC – lamina - structure</td>
</tr>
<tr>
<td></td>
<td>AC – lamina - cell shape</td>
</tr>
<tr>
<td></td>
<td>AC – lamina - cell number</td>
</tr>
<tr>
<td></td>
<td>AC – lamina - cloning</td>
</tr>
<tr>
<td></td>
<td>AC – lamina - PG content</td>
</tr>
<tr>
<td></td>
<td>AC – lamina - PG distribution</td>
</tr>
<tr>
<td></td>
<td>AC – mid layer - structure</td>
</tr>
<tr>
<td></td>
<td>AC – mid layer - cell shape</td>
</tr>
<tr>
<td></td>
<td>AC – mid layer - cell number</td>
</tr>
<tr>
<td></td>
<td>AC – mid layer - cloning</td>
</tr>
<tr>
<td></td>
<td>AC – mid layer - PG content</td>
</tr>
<tr>
<td></td>
<td>AC – mid layer - PG distribution</td>
</tr>
<tr>
<td></td>
<td>Tidemark (0 absent, 1 indistinct, 2 present, 3 multiple)</td>
</tr>
<tr>
<td></td>
<td>AC – calcified layer - structure</td>
</tr>
<tr>
<td></td>
<td>AC – calcified layer - cell shape</td>
</tr>
<tr>
<td></td>
<td>AC – calcified layer - cell number</td>
</tr>
<tr>
<td></td>
<td>AC – calcified layer - cloning</td>
</tr>
<tr>
<td></td>
<td>AC – calcified layer - PG content</td>
</tr>
<tr>
<td></td>
<td>AC – calcified layer - PG distribution</td>
</tr>
<tr>
<td></td>
<td>Cement line (0 absent, 1 indistinct, 2 present, 3 multiple)</td>
</tr>
<tr>
<td></td>
<td>Subchondral bone - structure</td>
</tr>
<tr>
<td></td>
<td>Subchondral bone - osteocytes</td>
</tr>
<tr>
<td></td>
<td>Subchondral bone - osteoblast activation</td>
</tr>
<tr>
<td></td>
<td>Subchondral bone - vascular invasion</td>
</tr>
<tr>
<td></td>
<td>Calcified cartilage in bone</td>
</tr>
<tr>
<td>Temporal surface</td>
<td>AC – lamina - structure</td>
</tr>
<tr>
<td></td>
<td>AC – lamina - cell shape</td>
</tr>
<tr>
<td></td>
<td>AC – lamina - cell number</td>
</tr>
<tr>
<td></td>
<td>AC – lamina - cloning</td>
</tr>
<tr>
<td></td>
<td>AC – lamina - PG content</td>
</tr>
<tr>
<td></td>
<td>AC – lamina - PG distribution</td>
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<tr>
<td></td>
<td>AC – mid layer - structure</td>
</tr>
<tr>
<td></td>
<td>AC – mid layer - cell shape</td>
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<td>AC – mid layer - cell number</td>
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<tr>
<td></td>
<td>AC – mid layer - PG distribution</td>
</tr>
<tr>
<td></td>
<td>Tidemark (0 absent, 1 indistinct, 2 present, 3 multiple)</td>
</tr>
<tr>
<td></td>
<td>AC – calcified layer - structure</td>
</tr>
<tr>
<td></td>
<td>AC – calcified layer - cell shape</td>
</tr>
</tbody>
</table>
Table 1. Other than tidemark and cement line, all parameters are scored: 0 = normal; 1 = slightly abnormal (<30% affected); 2 = moderately abnormal (30-70% affected); 3 = severely abnormal (>70% affected). AC – Articular Cartilage. PGs – Proteoglycans.

**Imaging analysis:** Imaging evaluation was performed and classified independently by 2 experienced radiologists (RS, LN) who were blinded to the intervention using the criteria outlined in Table 2.
Capítulo 7: Análise Histológica, Imagiológica e de Massa Corporal após Discectomia e Discopexia Bilateral num Estudo Pré-clínico Randomizado em Ocultação

<table>
<thead>
<tr>
<th>Condyle marrow</th>
<th>Change of underlying trabecular bone</th>
<th>This stage includes normal joint with no change of condyle trabecular bone</th>
<th>Sclerosis in less than a half of trabecular bone</th>
<th>Sclerosis in one half of trabecular bone</th>
<th>Sclerosis in all trabecular bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temporal marrow</td>
<td>Change of underlying trabecular bone</td>
<td>This stage includes normal joint with no change of temporal trabecular bone</td>
<td>Sclerosis in less than a half of trabecular bone</td>
<td>Sclerosis in one half of trabecular bone</td>
<td>Sclerosis in all trabecular bone</td>
</tr>
<tr>
<td>Calcification</td>
<td>Development of calcification across joint space</td>
<td>No calcification across joint space</td>
<td>Calcification in one third of joint surface</td>
<td>Calcification in two thirds of joint surface</td>
<td>Bony fusion across joint space</td>
</tr>
<tr>
<td>Global appreciation</td>
<td>Normal joint</td>
<td>In general mild changes</td>
<td>In general moderate changes</td>
<td>In general severe changes</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Imaging assessment.

**Body mass assessment**: Immediately after eating 150 gr of dry pellets the sheep were weighed. The body mass assessments were performed by 2 specially trained assessors who were not affiliated with the intervention. All animals had bilateral scar to reduce possible bias.

**Statistical analysis**

Statistical analyses were performed using either the Statistical Package for Social Sciences (IBM SPSS, version 22.0) or Statistics/data Analysis (STATA-corporation version 14.2). The histopathology scores for each parameter in each section of the 2 assessors were averaged, and following un-blinding the median scores (and score summations) for each treatment group were calculated. Differences between treatments were analysed by mixed ordinal logistic regression.
A one-way Analysis of Variance (ANOVA) was performed for cross-sectional analysis, in order to compare the outcome variables in the three levels of the IV before and after the random treatment group assignment. For longitudinal analysis a one-way ANOVA with repeated measures was performed taking as within-subjects effects observations after surgery (T1-T6) for bilateral discectomy, bilateral discopexy, and sham surgery conditions. Our primary analysis tested the effects of the independent variable (IV) surgical intervention (3 experimental conditions: 1 = bilateral discectomy; 2 = bilateral discopexy; 3 = sham surgery) using series pre-
test and post-test. As dependent variables we used body mass and imaging score for degenerative process. The body mass were measured 3 times in the pre-test for supporting invariance concerning the outcome measures before the clinical intervention (IV). Our secondary analysis (post-test) analysed the outcomes measuring 3 times, in 6 time-points, one per month at the same place, date and hour as in pre-test (Figure 3).

To analyze imaging results, non-parametric tests were performed attending to the sample size and the non-normality of the distribution for the majority of variables in each group (discectomy, discopexy, and sham surgery), Shapiro-Wilk test \((6) \leq .82, \ p \leq .091\). Kruskal-Wallis tests were performed for between group comparisons. Bonferroni test was used for post-hoc multiple comparisons. Partial eta squared \((\eta^2_p)\) and Cohen’s D were used for effect size calculations. Cohen (1988) defined effect sizes as "small, \(d = .2\)," "medium, \(d = .5\)," and "large, \(d = .8\)" (p. 25).

**RESULTS**

Descriptive baseline statistics of the animals studied are presented in Table 3.

<table>
<thead>
<tr>
<th>Sheep ID</th>
<th>Birth-date</th>
<th>Baseline mean of 3 measures</th>
<th>Allocation randomized process</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Body mass §</td>
<td></td>
</tr>
<tr>
<td>8298</td>
<td>11.01.11</td>
<td>56,0 Kg</td>
<td>Discopexy</td>
</tr>
<tr>
<td>9705</td>
<td>02.04.12</td>
<td>70,3 Kg</td>
<td>Discectomy</td>
</tr>
<tr>
<td>8264</td>
<td>19.07.10</td>
<td>56,3 Kg</td>
<td>Sham</td>
</tr>
<tr>
<td>9982</td>
<td>02.09.12</td>
<td>56,0 Kg</td>
<td>Discectomy</td>
</tr>
<tr>
<td>3969</td>
<td>30.10.09</td>
<td>57,0 Kg</td>
<td>Sham</td>
</tr>
<tr>
<td>8284</td>
<td>16.02.11</td>
<td>68,0 Kg</td>
<td>Discopexy</td>
</tr>
<tr>
<td>8267</td>
<td>13.07.10</td>
<td>75,7 Kg</td>
<td>Discectomy</td>
</tr>
<tr>
<td>9701</td>
<td>07.04.12</td>
<td>63,0 Kg</td>
<td>Discopexy</td>
</tr>
<tr>
<td>1903</td>
<td>25.12.12</td>
<td>52,0 Kg</td>
<td>Sham</td>
</tr>
</tbody>
</table>

* No significant differences between sheep in the reported characteristics were found at baseline, \(p > .10\).

§ Body mass is presented in kilograms.

**Table 3.** Baseline descriptive statistics.
1) Histologic results

The morphological appearance of the cartilage and bone in sham operated joints was consistent with that previously described as normal in the TMJ [56,146] (Figure 4Aa). The superficial half of the cartilage depth had a distinct laminar appearance, with sparse flattened cells and limited proteoglycan staining more intense with depth from the surface. Beneath this was a layer densely populated with cells that had a mesenchymal appearance, and more intense diffuse matrix proteoglycan staining. The deepest cartilage layer contained mature and/or hypertrophic chondrocytic cells often surrounded by a proteoglycan rich peri-cellular matrix but little or no interterritorial proteoglycan. A tidemark separating the upper two layers from the deepest cartilage layers could be observed in some sections, suggesting the lower zone was calcified. An indistinct cement line demarcated the subchondral bone which contained evenly distributed osteocytes in lacunae, and completely separated the cartilage from sparse marrow spaces lined by osteoblasts in the deeper bone. The synovium in sham-operated TMJ was similar to that in the knee joint in sheep [147] with a single lining layer of synoviocytes overlying a loose connective tissue with adipocytes and sparse fibroblasts and collagen.

A variety of pathological changes of varying severity were noted in discopexy and discectomy joints (Figure 4Ab-e). The mildest changes included cartilage thickening, slightly increased matrix and peri-cellular proteoglycan staining, increased cell density, vascular activation and invasion of the sub-chondral bone and calcified cartilage layer, with both the tidemark and cement line being more distinct (Figure 4Ab). Intermediate cartilage pathology was characterized by surface roughening/fibrillation, loss of typical laminar structure, a marked increase in interterritorial proteoglycan staining in all layers, cell cloning particularly in the upper zones, and further deep zone vascular invasion (Figure 4Ac). Further advancement of pathology was evident with erosion and loss of surface zone cartilage, decreased cell density in the mid zone but cloning in all layers, vascular invasion into the mid zone (Figure 4Ad), and ultimately complete loss of cartilage integrity and marked subchondral bone remodeling (Figure 4Ae). Accompanying the osteochondral changes, there was synovitis with hyperplasia of surface cells, sub-synovial fibrosis with loss of adipocytes and both peri-vascular and diffuse inflammatory cell (macrophages and lymphocytes) infiltration (not shown). Blinded scoring
demonstrated a significant increase in total median histopathology score in discectomy compared with both sham and discopexy groups (Fig 4B). This was driven by a significant increase in pathology in cartilage, bone and synovium in discectomy compared with sham-operated joints (Figure 4C). Discopexy joints did display some evidence of cartilage and synovial pathology in particular, but this was quite variable and thus did not reach statistical significance.

![Figure 4.](image)

**Figure 4.** (A) Representative images of toluidine blue stained sections of mandibular condyles from sheep with sham-operated (a) or discopexy/discectomy (b-e) TMJs. Total histopathology (B) and individual tissue (C) scores in Sham, Discopexy and Discectomy sheep (line = median; box = 25-75%, whiskers = 10-90%, dots = outside values).

2) Imaging results

The authors compared the nine outcomes between discectomy, discopexy, and sham surgery conditions (Table 2). For global appreciation differences were very high ($\eta^2_p$ corresponding to 90.8%, statistical power > .999). As demonstrated in
Table 4, there was statistical differences for all outcomes, excluding calcification (M = 0 and SD = 0 for all experimental conditions). Considering each outcome, differences were higher for shape, followed by condyle sclerosis, temporal sclerosis, condyle marrow, temporal erosion, condyle erosion, and, at last, temporal marrow. The effect size of the differences ranges from 43.4% to 90.8% for global appreciation. In Figure 5 we show a representative CT imaging of sham surgery group (Figure 5A), discopexy group (Figure 5B) and discopexy (Figure 5C).

![CT images](image_url)

**Figure 5.** A- Sham group, B- Discopexy group, C- Discectomy group.

<table>
<thead>
<tr>
<th>Surgery</th>
<th>Shape</th>
<th>Temporal erosion</th>
<th>Condyle Erosion</th>
<th>Condyle sclerosis</th>
<th>Temporal sclerosis</th>
<th>Condyle marrow</th>
<th>Temporal marrow</th>
<th>Calcification</th>
<th>Global appreciation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>SD</td>
<td>M</td>
<td>SD</td>
<td>M</td>
<td>SD</td>
<td>M</td>
<td>SD</td>
<td>M</td>
</tr>
<tr>
<td>Discectomy</td>
<td>2.83</td>
<td>0.41</td>
<td>1.50</td>
<td>0.84</td>
<td>13.83</td>
<td>1.10</td>
<td>2.33</td>
<td>0.82</td>
<td>1.50</td>
</tr>
<tr>
<td>Discopexy</td>
<td>1.17</td>
<td>0.41</td>
<td>0.67</td>
<td>0.52</td>
<td>9.33</td>
<td>0.41</td>
<td>1.33</td>
<td>0.52</td>
<td>10.50</td>
</tr>
<tr>
<td>Sham</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>4.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

X^2 (2) 16.22*** 9.30** 11.09**

η^2 0.96 0.46 0.68

Table 4. Means (M), standard-deviations (SD), Means Rank (MR), Kruskal Wallis Tests, power, and effect sizes for the outcomes.

Excluding the difference between discopexy and sham surgery for temporal erosion (Cohen’s d = 0.59), all the other differences were classified as a large effect size,
according to Cohen (Cohen’s d > 0.80). The larger differences are between discectomy and sham surgery (R^2 corresponding to 92.9% of degeneration in global appreciation), mainly due to shape (R^2 = 96.0%), condyle marrow (R^2 = 83.4%), and condyle sclerosis (R^2 = 80.1%); Condyle erosion and temporal marrow were the least affected, although with an effect size of R^2 of 50.3% and 50.8%, respectively; temporal sclerosis and temporal erosion showed effect sizes of R^2 of 71.1% and 62.3%, respectively. Discopexy also differed from sham surgery (R^2 corresponding to 80.3% of deterioration in global appreciation), although with lower effect sizes in comparison to the differences between discectomy and sham surgery, and only for shape (R^2 = 80.3%), condyle sclerosis (R^2 = 76.6%), and condyle marrow (R^2 = 56.7%) (Table 5 and Figure 6).
Capítulo 7: Análise Histológica, Imagiológica e de Massa Corporal após Discectomia e Discopexia Bilateral num Estudo Pré-clínico Randomizado em Ocultação

<table>
<thead>
<tr>
<th>Temporal marrow</th>
<th>Discopexy</th>
<th>Sham Surgery</th>
<th>1.33***</th>
<th>.34</th>
<th>.41 - 2.26</th>
<th>2.29</th>
<th>.753</th>
<th>.567</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.83</td>
<td>.44</td>
<td>-.36 - 2.03</td>
<td>0.89</td>
<td>.407</td>
<td>.166</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham Surgery</td>
<td>1.50***</td>
<td>.44</td>
<td>.31 - 2.69</td>
<td>2.03</td>
<td>.713</td>
<td>.508</td>
<td></td>
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<thead>
<tr>
<th>Global appreciation</th>
<th>Discopexy</th>
<th>Sham Surgery</th>
<th>0.67</th>
<th>.44</th>
<th>-.53 - 1.86</th>
<th>1.16</th>
<th>.500</th>
<th>.250</th>
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<tr>
<td>Discopexy</td>
<td>1.50***</td>
<td>.22</td>
<td>.91 - 2.09</td>
<td>3.20</td>
<td>.848</td>
<td>.719</td>
<td></td>
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<tr>
<td>Sham Surgery</td>
<td>2.67***</td>
<td>.22</td>
<td>2.08 - 3.26</td>
<td>7.26</td>
<td>.964</td>
<td>.929</td>
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Table 5. Mean differences between discectomy, discopexy, and sham surgery: Bonferroni test.

* p ≤ .05   ** p ≤ .01   *** p ≤ .001

Figure 6. Mean scores for the eight outcomes and the global appreciation.
3) Body mass results

Cross-sectional analysis. Statistical differences were not found in body mass in the pre-test (T0) and in all times for the post-test \((p > .10, \text{Table 6})\).

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<th>0</th>
<th></th>
<th>T1</th>
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<th>T4</th>
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<th>T5</th>
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<td>7.31</td>
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<td>67.33</td>
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<td>66.22</td>
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<td>54.33</td>
<td>8.14</td>
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<td>58.67</td>
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<tr>
<td>F(2, 6)</td>
<td>2.31</td>
<td>.435</td>
<td>1.12</td>
<td>.272</td>
<td>1.51</td>
<td>.335</td>
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<td>1.27</td>
<td>.297</td>
<td>1.00</td>
<td>.250</td>
<td>.86</td>
<td>.222</td>
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Table 6. Sheep body mass for T0 (pre-test) to T1-T6 (post-test): Descriptive and one-way ANOVA

In Figure 7 can be seen that in discectomy condition sheep lost weight from month 1 to month 4 and then recovery of their weight during months 5 and 6 after surgery.

Figure 7. Sheep weight for month 0 (pre-test) to months 1 to 6 (post-test) in discectomy, discopexy, and sham surgery.

Longitudinal analysis. A one-way ANOVA with repeated measures was performed taking as within-subjects effects months after surgery (T1-T6) for discectomy,
discopexy, and sham surgery. Statistically significant differences were found, $F(5, 10) = 9.69, 27.35$ and $8.07$, $p < .01$, $\eta^2_p = .829, .932$, and $.801$, $(1 – \beta) = .992, 1.00$, and $.977$ for discectomy, discopexy, and sham surgery respectively, showing that sheep recovered weight from T1 to T6. The tests of within-subjects contrasts identify that the increase happened from T4 to T5 both in discectomy ($p = .04$), discopexy ($p = .01$), and sham surgery ($p = .01$). Despite this increase, only in the discopexy and sham increased their weight over the pre-test in T5 and T6, $t(2) = -5.34$ and -5.00, $p < .04$. In discectomy and sham surgery conditions sheep didn’t exceed the weight they had in the pre-test.

DISCUSSION

This is the first temporomandibular preclinical study using a randomized, blinded design respecting ARRIVE guidelines. Using the suitable Black Merino sheep with age and gender selection, previously published protocol, sham control group and bilateral approach, the authors made an effort to reduce possible bias on results. In humans the TMJ cartilage is different from appendicular synovial joints [146], with the distinctly laminar fibrocartilage with sparse proteoglycan reminiscent of meniscus and annulus fibrosus of the intervertebral disc [148,149]. In sham-operated joints of Black Merino sheep the TMJ cartilage was histologically very similar to humans, supporting sheep as a good animal model. The rat [54] and goat [56] also have a typical TMJ fibrocartilage appearance with the distinct organized layers, while in the mouse [53,113] and rabbit [150] the laminar structure is less apparent.

The histopathology changes we found in the sheep TMJ after bilateral discectomy are consistent with other reports of induced osteoarthritis in various species, including mice [53], rats [54], rabbits [55] and goats [56]. Of particular interest is the increased proteoglycan and rounded cells and thickening of the cartilage commonly seen after discectomy. These changes in Black Merino sheep are similar to reports in other animals like mouse [53,66,151] and rat [54]. This is consistent with a chondroid metaplasia, potentially associated with the loss of the disc and increased direct loading in the TMJ cartilage. However, when this first protective phase fails under continued abnormal loading, the joint undergoes degeneration with cell death.
and cloning, surface erosion, subchondral bone changes and degeneration. This latter phase is well described and similar to that in the sheep knee joint following meniscectomy[111,152]. In the discopexy intervention group we preserved the TMJ capsule and the intra-articular environment. The result, as expected, had less severe histopathologic changes, because the disc remains interposed between the bony surfaces, dissipating the loading and protecting the TMJ cartilage. It is noteworthy that we also found more severe synovitis in discectomy compared with discopexy, indicating the inflammation it is not just a reaction to the arthrotomy but part of the OA process in the joint. The histopathological appearance of the synovitis in the TMJ was the same as that in sheep knee joints with OA [147]. However, given the underlying anatomical differences in cartilage, future studies should explore the molecular mechanisms that underlie TMJ OA pathology, to determine their similarities and differences with appendicular joints such as the knee [153]. Such studies could lead to progress in defining the pathophysiology and subsequently the management of TMJ degenerative disorders.

Radiographic morphologic changes followed discectomy was first reported by Boman in 1947, describing “flattening off the articular surface” [132]. Latter, in 1985 similar conclusions were obtained with condylar flattening and sclerosis after unilateral discectomy where no osteophytes but severe damage are described [135]. In 1988, in a 33.8 years post discectomy condyle flattening and sclerosis were the most common radiographic findings [137]. In our study we also observed severe morphological changes after bilateral discectomy. Most statistical differences were noted in shape and condyle sclerosis, corresponding to other author’s clinical findings. While the human condyle is convex and tends to flatten after discectomy, the sheep condyle is normally flat and appears to change to a more convex form after discectomy (Figure 5.C). Nevertheless, condyle sclerosis was observed in all joints after bilateral discectomy ($R^2 = 80.1\%$), and we also detected change in underlying trabecular bone (condyle bone marrow). Cortical breakdown characterized by an initial destructive phase was reported by Agerber and Lundberg in the first 6 months post-discectomy [154]. Some authors suggest that these changes can occur if loading is not controlled during the first 6 months after discectomy [134]. Other authors raised the question of whether the lytic condylar
process is precipitated by the discectomy or the overloading, since the contralateral non operated joint has similar morphologic changes [135,138,154,155]. We could not find and reports on temporal bone evaluation in patients, but our study suggests they are similar to those in the condyle. Yaillen, in 1979 described bony ankylosis between the condyle and temporal bone 1 year after unilateral discectomy in *Macaca fascicularis* [156]. Latter, Bjornland after 6 months unilateral discectomy found fibrous ankylosis [45]. In TEMPOJIMS, 6 months after bilateral discectomy we did not find any intra-articular calcification, and while with CT we cannot exclude fibrous ankylosis this was not evident histologically. We also report significant osteophyte formation, rarely described in previously studies, which may be due to imaging limitations of radiography and arthrography compared with CT.

We are not aware of any clinical or preclinical studies that have evaluated imaging after discopexy. Our results showed that TMJ open surgery is not innocuous, resulting in a mild to moderate changes in global remodeling. The condyle is more affected than the temporal bone and only for shape ($R^2 = 80.3\%$), condyle sclerosis ($R^2 = 76.6\%$), and condyle marrow ($R^2 = 56.7\%$) were significant changes detected. We lack other studies to compare our results. These results are the minimum expected in TEMPOJIMS phase 2, were a surgical intervention is needed to remove the TMJ disc and insert a temporomandibular interpositional implant.

In other diseases like rheumatoid arthritis [157], cancer [158], HIV [159] and surgical interventions like gastric sleeve [160] body mass has been used as a valuable outcome to evaluate progress of disease and intervention success. In TMJ this outcome has rarely been attempted. Goss (1999) reported 4% of body mass lost in 60% of the animals 3 months after unilateral discectomy with condyle and temporal surfaces removal [144]. In a study in mice, after partial discectomy no significant losses or gains in the body weight of the experimental or control mice was seen [113]. We have found 5.2% body mass lost 3 months after bilateral discectomy (all occurring in the first month) but with full recovery at 6 months follow-up. In contrast, the discopexy and sham surgery sheep increased body weight (mostly in T4-T6) finishing the study 8% and 8.2% respectively, more the baseline. The bilateral discopexy body mass change was the same as the sham surgery group, is consistent with the limited TMJ pathology. The evaluation of body mass was also a welfare control measure related to healthy and well feed, respecting the 3Rs
principle (replacement, reduction, or refinement) [161]. Potential animals suffering would be reported to the veterinary team if a 10% body mass loss were detected in any animal.

Potential limitations of TEMPOJIMS study include the relatively small sample size. This pilot investigation demonstrates it is feasible using this study design to conduct preclinical trials in TMJ treatment in Black Merino Sheep. Bilateral discopexy in a healthy TMJ is not an innocuous intervention, resulting in variable cartilage and synovial pathology along with imaging changes. In contrast, bilateral discectomy induced severe TMJ changes detected with both imaging and histopathologic analysis. No fibrous or bony ankylosis was detected over the 6-month time course which would limit model utility. Beyond expected cartilage and bone changes, synovitis was shown to be part of the osteoarthritis process, providing a new outcome measure and therapeutic target. While the animals were able of recover the baseline body mass in 6 months consistent with it being an ethically appropriate model, the early weight loss may also be modifiable outcome for therapeutic trials. This study has reinforced that TMJ cartilage is different from appendicular synovial joints, and as such may require unique therapeutic approaches. We hope that the TEMPOJIMS phase 1 results reported here, will motivate other investigators to study potential biomaterials to substitute the TMJ disc, and will help in consistent study design and evaluation to improve interpretation of findings and translation to new treatments.

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Author contributions
DAF, PM, NA and DS designed the study, DAF, FS, FM and RG performed all surgical procedures, SC, BC, MP, FC contributed with the veterinary support, CL, SS and MS contributed to the histological analysis, RS, LN and IC contributed to imaging analysis, PM, MA, CM, YW, EJ and JG contributed to TEMPOJIMS phase
2, LM and DAF contributed to analysis and interpretation of data, DAF, CL and PM drafted the article and revising it. CL, MS and PM revised the article and finally approved the final version of the article to be published.

Role of the funding source
This work has been granted by Portuguese Grunenthal Foundation and by Secção Regional Oeste da Ordem dos Médicos.

Conflict of interest
There are no conflicts to declare.
CAPÍTULO 8. DISCUSSÃO GERAL E CONCLUSÕES

8.1. DISCUSSÃO GERAL

O principal objetivo da presente investigação foi contribuir para o avanço científico na compreensão do impacto morfofuncional das técnicas cirúrgicas, discectomia e discopexia. Os principais resultados do nosso estudo demonstraram que a discectomia induziu um dano degenerativo severo na ATM com alterações estatisticamente significativas nas análises histológica e radiológica. Após 6 meses, não se verificaram alterações significativas na análise da cinemática ruminatória, na análise do tempo de mastigação inicial e no peso corporal. A discopexia não induziu danos relevantes na ATM e não se verificaram alterações estatisticamente significativas em nenhuma das variáveis estudadas.

Na atualidade, é globalmente reconhecida a importância da adequada delineação dos desenhos de estudo para garantir elevada qualidade de investigação. Porém, com frequência, essa premissa é descurada [162], inibindo a formulação de conclusões coerentes e robustas. O termo desenho de estudo, ou desenho experimental, pode ter várias interpretações na literatura, sendo muitas vezes associado ao tipo de estudo utilizado para planificação de uma determinada investigação. Todavia, neste, deve estar implícito todo o protocolo e as estratégias usadas para reduzir o risco de enviesamento dos resultados [163]. Sendo os ensaios clínicos randomizados e ocultos a referência padrão para investigação clínica em humanos, existem dois protocolos de referência, com orientações para os estudos: o CONSORT [163] e o SPIRIT [61]. Estes protocolos contêm recomendações e listas de verificação a adotar, e a sua elaboração foi fundamentada pela necessidade de homogeneizar e clarificar os procedimentos na tentativa de promover ensaios clínicos de qualidade, transparentes, reduzindo o risco de enviesamento dos resultados.

Em 2010, foi possível verificar que em 271 estudos pré-clínicos, apenas 59% indicavam: os objetivos e a hipótese de investigação do estudo, o número de animais, a raça, a espécie, o gênero e a idade. Verificou-se ainda que apenas 13%
dos estudos foram randomizados e 14% ocultos [58]. Estes resultados são preocupantes e demonstram o panorama atual que enfrenta a investigação pré-clínica. Precisamente com o propósito de garantir qualidade superior nos estudos pré-clínicos e progredir na medicina de translação, foram elaboradas recomendações standard – as ARRIVE guidelines [58]. Assim, pretende-se que os estudos pré-clínicos modernos sejam rigorosos, transparentes e que respeitem a regra dos três R: Replacement, Refinement and Reduction of Animals in Research e as ARRIVE guidelines [58]. Quando analisados os estudos pré-clínicos mais relevantes na área da ATM (n = 32) foi possível verificar que, além das incertezas em relação ao animal mais apropriado a utilizar, nenhum estudo respeitava as ARRIVE guidelines. Foi objetivo do presente trabalho alterar o paradigma dos estudos pré-clínicos na área da ATM, propondo um desenho de estudo inovador, rigoroso, randomizado e oculto (capítulo 5), recorrendo para isso ao modelo animal mais adequado – a ovelha Black Merino (capítulo 3).

Relativamente à problemática do modelo animal mais adequado para investigação na área de regeneração intra-articular e desenvolvimento de materiais de interposição articular, a literatura não é clara nem consensual. Se por um lado o rato parece ser um modelo adequado, para estudos pré-clínicos relacionados com investigação de mecanismos inflamatórios e dor na ATM [164–169], animais de maior porte serão mais apropriados para uma investigação na área de desenvolvimento de técnicas cirúrgicas minimamente invasivas, materiais de interposição e próteses da ATM.

anatómico. Estes resultados levaram os autores a concluir que o macaco Rhesus seria o modelo animal mais adequado para conduzir investigação pré-clínica na ATM [173]. No entanto, por motivos éticos e económicos, o uso dos primatas foi abandonado no início do século XX.

Durante o mesmo período temporal, uma outra linha de investigação apontava para a utilização do porco como animal experimental mais adequado para investigação na ATM [174], seguindo-se uma série de estudos descritivos post mortem sobre a ATM e o disco deste animal [175–191]. De facto, a elevada semelhança com a articulação humana, levou a que durante os últimos 20 anos o porco doméstico tenha sido considerado o gold standard da investigação pré-clínica em ATM. Contudo, quando se reveem os estudos pré-clínicos in vivo, é possível verificar que poucos (n = 2) foram os estudos conduzidos no porco doméstico [192,193]. Optou-se, frequentemente, por recorrer ao mini-pig [69,194–199]. Assim, é possível verificar que, para investigações post mortem no domínio do disco, foi recorrente a utilização do porco doméstico, enquanto que para estudos in vivo deu-se primazia ao uso do mini-pig; este último menos estudado, assumindo-se empiricamente que a anatomia seria semelhante à do porco doméstico. Já em 2013, foi relatado que nem todas as estruturas da ATM do porco são semelhantes às do humano, sugerindo reflexão sobre a continuidade da sua utilização no futuro [200].

Existem ainda referências a outros modelos para estudos pré-clínicos. Por exemplo, o cão foi recentemente utilizado para investigação de materiais de interposição na ATM [65,201], embora alguns estudos evidenciem diferenças anatómicas significativas entre a ATM dos caninos, do porco e do humano [202]. Adicionalmente, as sobrecargas articulares próprias dos carnívoros [82] podem ter um impacto negativo na interpretação e transferência dos resultados.

O grupo de Goss et al foi quem mais estudos in vivo realizou recorrendo à ovelha [50,86–89,145,203–216]. Seguiram-se outros estudos pré-clínicos nessa linha de investigação, com sucesso [217–220]. No entanto, ao contrário do que acontece com o porco em que existem múltiplos estudos descritivos e escassos in vivo, na ovelha não se encontra na literatura nenhum estudo que avalie a anatomia, a
Capítulo 8: Discussão Geral e Conclusões

biomecânica e a histologia da ATM. Foi esse o mote para a realização do capítulo 3, obtendo-se uma descrição da anatomia da ovelha adulta Black Merino, da histologia e biomecânica do seu disco articular. Após esta descrição rigorosa e, cumulativamente à experiência de outros autores com este modelo, foi possível concluir que a ovelha deve ser o animal modelo para investigação experimental na ATM, sugerindo o nosso grupo a raça Black Merino para diminuir a variabilidade biológica inter e intra-resultados.

Considerando a correta seleção do modelo animal (capítulo 3), é fundamental proceder a uma adequada seleção das variáveis a serem estudadas. O protocolo proposto (capítulo 5) define essas variáveis, como resultado das caraterizações obtidas (capítulo 3 e 4). Em estudos anteriores, ao tentar avançar no desenvolvimento de materiais de interposição na ATM, não foi tida em consideração a morfologia do disco nativo. No presente trabalho, e ao perspetivar futuros avanços no desenvolvimento de biomateriais, foi nossa intenção caracterizar o disco da ATM da ovelha Black Merino, recorrendo a tecnologia de scanning 3D. Esta metodologia, amplamente utilizada nas mais diversas áreas da engenharia, permitiu obter uma replicação exata da morfologia do disco no seu estado natural. No entanto, além dos parâmetros morfológicos, revelou-se determinante compreender os parâmetros cinemáticos da ruminação, tempo de mastigação (capítulo 6), parâmetros imagiológicos e histológicos (capítulo 7).

Para controlar o sucesso ou insucesso das intervenções cirúrgicas em doentes com DTM, deverão ser analisadas, cumulativamente, a dor e a abertura oral. No entanto, não se tentou, no passado, extrapolar as considerações destas duas variáveis em estudos pré-clínicos na ATM. Para o presente trabalho, testou-se a hipótese de um animal com dor na ATM demorar mais tempo para ingerir uma determinada quantidade de ração. Assim, foi possível inferir que as ovelhas sujeitas a discectomia, demorando mais tempo a comer 150gr de ração, foram as únicas a alterar o seu comportamento mastigatório pela indução da dor numa fase inicial. A ausência de estudos com este tipo de análise não permite efetuar comparações, reforçando a importância de incluir a avaliação biomecânica nas investigações em
torno da ATM (capítulo 3 e capítulo 4), e de garantir um apropriado procedimento experimental.

Na literatura encontramos estudos em que na remoção unilateral do disco é usado o lado contralateral como grupo de controlo [48,53,64,65,68,113-117]. Todavia, após intervenção unilateral há uma sobrecarga do lado contralateral [110], o que pode enviesar os resultados. A metodologia utilizada no presente trabalho pretendeu ultrapassar essa limitação (capítulo 5), realizando a discectomia bilateral no grupo 1, a discopexia bilateral no grupo 2, e mantendo um grupo de controlo (grupo 3). O grupo 1 teve como objetivo primário analisar o impacto da discectomia bilateral na ATM da ovelha. O grupo 2, ao qual foi seccionado o disco posteriormente e suturado na sua posição inicial, teve como objetivo perceber o efeito da intervenção na articulação. O grupo 3 (grupo controlo) funcionou como placebo, onde se realizou uma intervenção cirúrgica sob anestesia geral, sem invadir o espaço articular.

Este trabalho foi pioneiro na área da ATM, ao usar, pela primeira vez, um protocolo (capítulo 5) de randomização, ocultação, intervenção bilateral, com variáveis histológicas, radiológicas (capítulo 7), cinemáticas e funcionais (capítulo 6) no modelo animal mais adequado (capítulo 3 e capítulo 4), respeitando as ARRIVE guidelines.

Por último, esta investigação reforçou a importância das equipas multidisciplinares para responder a desafios na área da medicina regenerativa. Como tal, para a realização deste trabalho estiveram envolvidas equipas: (i) da biomecânica e bioengenharia, que aportaram um ganho significativo na compreensão da biomecânica da articulação e na caracterização da cinemática ruminatória; (ii) da histologia, que permitiram consolidar o impacto histológico da discectomia e da discopexia, assim como consolidar o conhecimento histológico da ATM normal; (iii) da imagiologia que permitiram uma avaliação rigorosa da ATM; (iv) da medicina veterinária que permitiram conduzir este estudo respeitando as ARRIVE guidelines; (v) do departamento de investigação clínica com experiência em desenhos de investigação que permitiu aperfeiçoar o desenho para estudos da ATM; (vi) e de
cirurgiões experientes no tratamento de disfunções da ATM que conduziram e orientaram esta investigação para o progresso do tratamento dos doentes com DTM severa.

8.2. IMPLICAÇÕES PARA FUTURA INVESTIGAÇÃO E CLÍNICA

Os resultados obtidos após discectomia mostraram um processo degenerativo severo imagiológico e histológico, sem uma repercussão significativa na função mastigatória e ruminatória da ovelha. Estes resultados demonstraram similaridades com os apresentados na investigação pós discectomia em humanos [138,221]. No entanto, esse resultado pode estar associado a uma sobrecarga contralateral, pois, no humano a discectomia bilateral tem resultados menos satisfatórios que a discectomia unilateral [45], não sendo por isso aconselhada. A discectomia unilateral apresenta-se como uma técnica com bons resultados clínicos em estudos a 20 anos. Contudo são necessários mais estudos para avaliar se esses resultados não estão associados a uma sobrecarga contralateral [63]. Deverá dar-se continuidade à linha de investigação que tenta, já há vários anos, reproduzir um biomaterial compatível com o disco articular nativo.

A discopexia bilateral da ATM causou alterações minor, tanto na análise histológica como radiológica. Na análise da função ruminatória e mastigatória este grupo teve um comportamento semelhante ao grupo controlo, sugerindo ser menos invasiva e agressiva que a discectomia. No entanto, no humano esta técnica está associada a muitas recidivas. Num próximo estudo para testar na ATM possíveis biomateriais, a intervenção cirúrgica não será responsável por induzir danos significativos, devendo este grupo ser usado como controlo. O modelo animal proposto (capítulo 3) e o desenho de estudo já testado (capítulo 4) podem representar uma mais-valia, acrescentando rigor e transparência dos resultados obtidos.
8.3. CONSIDERAÇÕES FINAIS

- A ovelha *Black Merino* é um animal que apresenta as características apropriadas para ser utilizado em estudos da ATM, por evidenciar uma anatomia cirúrgica semelhante, e o disco exibir morfologia, biomecânica e histologia comparáveis. Por ser um animal ruminante poderá favorecer estudos posteriores para investigar potenciais biomateriais ou próteses da ATM que permitirão testar a fadiga do material convenientemente.

- O desenho de estudo proposto randomizado, oculto, com intervenções cirúrgicas bilaterais verificou-se exequível e reprodutível para futuras investigações na área da ATM em ovelha *Black Merino*.

- Os *outcomes* selecionados: *outcome* primário – análise histológica, *outcomes* secundários - análise radiológica, análise da mastigação, ruminação e peso corporal foram conseguidos e permitiram ampliar o conhecimento sobre o efeito de cada intervenção em cada um destes *outcomes*.

- Na análise histológica verificou-se que, 6 meses após a cirurgia, o grupo controle apresentava características iguais às descritas como normais no capítulo 1. O grupo da discectomia apresentava diferenças estatisticamente significativas com: alterações osteocondrais, hiperplasia das células sinoviais, fibrose subsinovial e infiltração de células inflamatórias (macrófagos e linfócitos). O grupo da discopexia apresentava alterações patológicas na cartilagem e na membrana sinovial, mas sem significado estatístico.

- Na análise radiológica encontraram-se alterações estatisticamente significativas no grupo da discectomia com degeneração severa, mas sem progressão para anquilose. Na discopexia houve alterações ligeiras sem significado estatístico. O grupo controlo não apresentou alterações.

- As ovelhas do grupo da discectomia e discopexia perderam peso após a cirurgia. O grupo da discectomia conseguiu recuperar o peso inicial após 6
meses. O grupo controlo (+8.2%) e da discopexia (+8%) superaram o peso pré-operatório. Não se encontraram diferenças estatisticamente significativas.

- A avaliação do tempo de mastigação mostrou diferenças estatisticamente significativas, no 1º mês pós cirurgia, para a discectomia e discopexia. Os animais conseguiram recuperar após o 1º mês.

- A análise geral da cinemática ruminatória permitiu observar que uma ovelha do grupo da discectomia não teve movimentos de ruminação no mês 1 e 2 pós cirurgia. A análise da área de ruminação mostrou ser menor no grupo pós discectomia, sem significado estatístico.

- A bioengenharia de tecidos poderá desempenhar um papel significativo na regeneração do disco articular, melhorando as técnicas cirúrgicas atuais, nomeadamente a discectomia.
AGRADECIMENTOS

A presente tese representa um percurso clínico e científico que teve sempre um objetivo comum: contribuir para um progresso significativo no meio clínico e científico dos doentes com DTM. Por esta razão, endereço o meu primeiro agradecimento aos meus doentes, que sempre acreditaram em mim, que foram a verdadeira força motriz para este projeto se tornar real.

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Esta dissertação reuniu diversos ramos da ciência, medicina, medicina dentária, medicina veterinária, bioengenharia, ciências biomédicas. Desta natureza multidisciplinar resulta a necessidade de reconhecer publicamente numerosas influências e contribuições:

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• Segundo prémio no concurso 9º concurso de ideias de negócio de Cascais na categoria health medical devices ideas - DNA ideias com o projeto TEMPOJIMS. 11 de Maio de 2015

• Bolsa da Fundação Grünenthal para jovem investigador na área da dor. 13 de março de 2015
REFERÊNCIAS

Referências


Referências


Introduction

To improve human health, scientific discoveries and technologies must be translated into practical applications. Such advances classically begin with basic research and then progress to the clinical level. Inherent to the development of new technologies is the role of preclinical trials using animal models. Although no animal model can fully replicate human conditions, animal models are key in the evaluation of mechanisms of disease, testing new technologies and applying new procedures. Temporomandibular joint (TMJ) is the joint most frequently used in the human body. TMJ opens and closes 1500–2000 times daily and is essential for everyday functions of the mouth such as mastication, speech, deglutition, yawning and snoring involving special mandibulomandibular and temporomandibular joint (TMJ) functions. It is an essential component in the normal TMJ and has the following functions: it distributes the intra-articular load, stabilizes the joints during translation and decreases the wear of the articular surface [2, 3]. TMJ disc is displaced, malformed or damaged, can induce pathologic processes of internal derangement and/or osteoarthritis [4, 5]. Cerebrospinal fluid (CSF) is the ideal medium for this research due to its homogenous nature. The TMJ is complex and diagnostically challenging, especially in cases of internal derangement. Analysis of CSF is crucial for detection and diagnosis of pathologies. Therefore, the development of new technologies for scaffolds engineering regarding TMJ disc is growing [9–11] and the ideal
animal model for TMJ research should be well characterized. The choice of an animal for experimental design is not straightforward. Due to physiological and anatomical differences between the human TMJ and that of experimental animals, there is no animal model that is valid per se. TMJ is a cardinal feature that defines the class Mammalia and separates mammals from other vertebrates [12]. TKU shows remarkable morphological and functional variation between different species, reflecting not only the great mammalian adaptation to feeding mechanisms but also different biomechanical behavior [13]. The morphological variations are either correlates of loading (e.g. size of articular surfaces) or movement (e.g. orientation of the joint), or both. Loading of the TMJ is a reaction force arising from the contraction of masticatory muscles; its magnitude depends strongly on the position of the bite point relative to the muscle action line [14]. Many commonly used laboratory animals, especially rodents, fall in the category of minimal TMJ loading, especially during chewing. In contrast, carnivores such as dogs sustain TMJ loads that are higher than those of primates [15]. Opening of the jaw usually involves a combination of rotation and forward sliding (translation), but some carnivores have lost the ability to slide and some specialized antelopes instead use a rotation around the long axis of the curved mandible [13]. The most extreme evolutionary variants include:

- loss of the synovial cavity in some baleen whales;
- loss (or possibly primitive absence) of the disc in monotremes, some marsupials, and some edentates (anteaters and sloths);
- variations in the orientation of the joint cavity from sagittal (many rodents) to transverse (many carnivores);
- reversal of the usual convex/concave relationship so that the processus condylaris becomes the female element (many artiodactyl ungulates such as sheep and cattle).

In addition, the relative size of the joint is exceedingly variable. Sheep, rabbit, and monkey disc defect models in many studies [16–23]. Monkey model is rarely used in recent years, considering the high cost, difficult surgical operation and ethical approval. Rabbit is an excellent option for TMJ disc anterior dislocation studies but the small size of TMJ increases the difficulty for surgical approach and disc manipulation. The authors agree with other studies considering sheep is a valid option for TMJ studies due to TMJ size, processus condylaris and fossa mandibularis shape, disc size, morphology and attachment [12]. However, a deep biochemical and biomechanical characterization of the sheep TMJ is lacking in the available literature. Hence, the aim of the present study was to examine the morphological, histological and biomechanical properties of TMJ discs extracted from sheep (Ovis aries). It was hypothesized that these discs would present high similarity with available data on human TMJ.

Materials and methods

The material used for this study was obtained from sheep slaughtered for meat consumption. A total of 15 heads from Black Merino female sheep, 40 to 50 kg, were used: 6 for morphological characterization, 4 for histological characterization and 5 for biomechanical testing. One of the major requirements for this study was to use fresh TMJ discs; for that reason a team of certified surgeons was available 5 days weekly to collect fresh TMJs up to a maximum of 5 hours after death. Regarding the animal ethical considerations, the present study design was approved by the Portuguese National Authority for Animal Health.

Morphological characterization

For morphological characterization, 12 fresh TMJ discs were collected from six sheep heads. A surgical dissection was performed exposing and identifying TMJ anatomical structures. All muscular attachments were removed to obtain clean TMJ discs. Discs were submersed for 5 minutes in a Colcemid solution, an extremely fast-acting fluorintant, designed to rapidly strengthen 3D-printed parts. This submersion was essential to maintain the correct morphology for the 3D scanning. A white light 3D scanning system (Steinbichler – COMET 5*) and the appropriate software were used to replicate the discs in a 3D virtual model. Once the discs removed, two of the skulls were boiled in water (120 °C) for 2h to allow the procurement of complete clean crania.

Histological characterization

Four sheep heads were used to conduct the histological investigation. The TMJ were removed using a necropsy bone oscillatory saw according to the following anatomic references: cranial – cranial aspect of processus coronoides in the section of the arcus zygomaticus, caudal – external to the meatus acusticus. The dorsal reference was established to the squamous temporal bone. The ventral reference was 2 cm ventral to the meatus acusticus in the zone of angulus stylohyoideus.

The joints were fixed in 10% buffered formalin for ten days. Decalcification was obtained by immersion in 10% formic acid for three weeks, after which the articulations were cut sagittally and transversally through the whole processus condylaris. After intensive washing the fragments were submitted to routine tissue processing with paraffin embedding. Four-micron sections were stained with hema-toxalin and eosin (H&E) and with Orcein to show elastic fibers in the disc. Digital images were obtained with an Olympus DP21 camera.

Biomechanical testing

Five sheep heads were used for biomechanical studies. TMJ discs were removed and immersed in a saline solution for transport up to the bioengineering facilities (1 hour maximum). All muscular attachments and ligaments were removed to obtain a clean fibrocartilaginous disc. Ten clean discs were obtained but one was excluded due to surgical damaging. Consequently, 9 discs were randomized in 3 groups and tested in different mechanical tests: Tensile modulus (E), tensile strength and elongation were tested in: anteroposterior tests (APT) and mediolateral tests (MDT).
Figure 1  Direction of loading on: (A) anteroposterior and (B) mediolateral tensile tests. The dotted line represents the limit used to fix temporomandibular joint (TMJ) discs in grips. Tension tests used a strain rate of 0.1 mm/min with an initial distance between grips (LO) of 2 mm. All tests were conducted on a Zwick 2100 strength-testing machine (Zwick GmbH & Co. KG, Germany) equipped with a 10 kN load cell. For the compression tests the same rate was applied.

Compliance tests (CT) were performed using a stress-strain tests. In case of anteroposterior tensile test, during loading, the TMJ discs were stretched in the direction represented on Fig. 1A, while in mediolateral tensile test the direction of stretching was as shown on Fig. 1B.

Results

Morphological characterization

In the sheep heads studied, the TMJ was located, as expected, in the posterior segment of the side of the face, cranioventral to the external meatus acusticus, being a diarthrodial, bicondylar joint that allows normal opening and closing of the mandible. It comprised the superior articulating face, the fossa mandibularis of temporal bone, and the processus condylaris, as the inferior articulating surface (Figs. 2 and 6). A protruding processus coronoides was noted (Fig. 3).

The superior articulating surface (fossa mandibularis) was located in the inferior zone of temporal bone, lateral of foramen ovale and anterior to the external meatus acusticus. The fossa mandibularis was anteroposterior larger than mediolateral with a convexity downwards. The inferior articulating surface (Fig. 3) is represented by the processus condylaris, with ellipsoidal shape with the longer axis in the mediolateral position, the mean measures being 23.47 mm long (σ = 0.87) and 8.32 mm wide (σ = 1.54). The processus...
condylar lars was mediolateral concave. The fossa mandibularis receives the processus condylaris.

With an easy surgical approach the authors located the fibrocartilaginous joint disc interposed between the fossa mandibularis and the processus condylaris (Fig. 4). This disc separates an upper joint cavity from a lower one. The first was consistently larger than the second. The bony structures were coated with cartilage more evident in the processus condylaris. In the ewes studied, the joint disc had an elliptical shape, being substantially thinner in the center than at the periphery. TMJ disc regions are commonly classified as anterior band, posterior band, and intermediate zone (Fig. 5). The intermediate zone exhibits differences from its lateral to medial aspects, being often subdivided into lateral, medial, and central region. The bands discs are thicker than the intermediate zone.

The mean length and width of the 12 analyzed fresh TMJ discs were 21.33 mm (± 1.33) and 11.49 mm (± 0.62), respectively. Anterior and posterior band thicknesses were 1.05 mm (± 0.07) and 1.27 mm (± 0.04), respectively. Mean central thickness was 0.76 mm (± 0.09).

The same measures obtained from the 3D virtual models were totally similar to the ones registered in the fresh discs. An important report and consistent with all TMJ was the presence of viscous fluid in upper and lower compartment. This fluid was not analyzed.

Histological characterization

The histological study of the sheep TMJ revealed that the articular disc was attached anteriorly and posteriorly to

Figure 3  Articular surfaces of the temporomandibular joint (TMJ). A. Superior articular surface: (1) fossa mandibularis, (2) arcus zygomaticus, (3) foramen ovale. B. Inferior articular surface: (1) processus condylaris, (2) processus coronoideus, (3) incisura mandibulae, (4) foramen mandibulae. P: posterior; A: anterior; M: medial; L: lateral.

Surfaces articulaires de l’articulation temporo-mandibulaire. A. Surface articulaire supérieure : (1) fossa mandibularis, (2) arcus zygomaticus, (3) foramen ovale. B. Surface articulaire inférieure : (1) processus condylaris, (2) processus coronoideus, (3) incisura mandibularis, (4) foramen mandibulae. P : postérieur ; A : antérieur ; M : médial ; L : latéral.

Figure 4  View of the right temporomandibular joint (TMJ). To improve visualization the authors pulled down the processus condylaris. (1) Cartilage surface of fossa mandibularis in the upper joint compartment, (2) temporomandibular joint disc, (3) retrodiscal tissue, (4) muscle pterygoideus lateralis (5) cartilage surface of the processus condylaris (6) external meatus acusticus. P: posterior; A: anterior; M: medial; L: lateral.

the articular capsule composed of fibrous tissue. Both the fossa mandibularis and the processus condylaris surfaces were covered by a fibrocartilaginous layer. However, the fibrocartilaginous layer covering the processus condylaris was considerably thicker than the layer covering the fossa mandibularis (Fig. 4).

The central thin part of the disc consisted of scattered fibroblasts and densely packed, thick collagen fiber bundles arranged mainly in an anteroposterior direction. The collagens fibers were not straight but showed evidence of a wavy outline. The anterior and posterior disc portions were in turn occupied by collagen fiber bundles with diverse orientations (Fig. 7). In some areas, these two portions showed chondrocyte-like cells residing in lacunae distributed among less compact collagen fibers (Fig. 7). Each lacuna was surrounded by minimal amount of amorphous matrix. The posterior band blended, in the retrodiscal space, with loose connective tissue with profuse blood and nerve supply. A few small caliber blood vessels, surrounded by loose connective tissue, were observed in all parts of the disc.

Table 1 Length, width and thickness of the 9 discs tested.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dimensions (mm)</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length</td>
<td>Width</td>
</tr>
<tr>
<td>APT1</td>
<td>22.71</td>
<td>11.06</td>
</tr>
<tr>
<td>APT2</td>
<td>23.89</td>
<td>10.69</td>
</tr>
<tr>
<td>APT3</td>
<td>20.43</td>
<td>11.29</td>
</tr>
<tr>
<td>MDT1</td>
<td>19.60</td>
<td>12.63</td>
</tr>
<tr>
<td>MDT2</td>
<td>20.57</td>
<td>10.56</td>
</tr>
<tr>
<td>MDT3</td>
<td>20.05</td>
<td>11.39</td>
</tr>
<tr>
<td>CT1</td>
<td>20.75</td>
<td>10.07</td>
</tr>
<tr>
<td>CT2</td>
<td>20.49</td>
<td>11.93</td>
</tr>
<tr>
<td>CT3</td>
<td>19.94</td>
<td>10.44</td>
</tr>
</tbody>
</table>

AP: anteroposterior tests; MDT: mediolateral tests; CT: compression tests.
Sheep and temporomandibular joint

The obtained results demonstrated that the tensile modulus of mediolateral tensile tests is higher than anteroposterior tensile tests, as well as the tensile strength and elongation at break (Figs. 10 and 11).

In Table 2 the results obtained for the tested discs for tensile modulus, tensile strength and elongation at break are summarized.

Mechanical testing under compression was performed to evaluate the macro-mechanical performances of the TMJ discs. Fig. 12 demonstrates the compressive stress-strain curves of the tested discs.

The TMJ discs presented a compressive modulus (E) of 446.41 ± 5.16 MPa and their maximum stress value (σmax) was 18.87 ± 1.33 MPa.

Discussion

TMJ disc is a specialized fibrocartilaginous tissue, located between the processus condylaris and the fossa mandibularis [2, 4, 24] as shown in our sheep morphologic characterization. In humans TMJ disc has an elliptical perimeter, thinner in the center than on periphery. Disc periphery acts like a ring structure supporting the central zone. The same was observed in sheep disc morphology. The functions of the TMJ disc are:

- to improve the fit between bony surfaces;
- to provide stability during mandibular movements;
- to distribute masticatory forces [25].

This capacity is due to the high concentration of collagen fibers. This ring structure around the disc is an important structural aspect to support disc connections. The connection area is rich in elastic fibers, which is essential to disc mobility in the joint. As it was shown in the morphological characterization of the sheep TMJ, this anatomical structure revealed several similar characteristics with the TMJ in humans, including the mediolateral diameter being larger than the anteroposterior, the long axis of the processus condylaris directing backwards, and larger anterior condylar slope. One of the main differences is the concave form of the mediolateral processus condylaris that is convex in humans. The processus condylaris forms a small anteroposterior and mediolateral depression (Fig. 7). Instead, in the anterior and posterior disc portions, elastic fibers showed a reticular distribution among collagen fibers and chondrocyte-like cells (Fig. 9).

Biomechanical characterization

In Table 1, the measures of the discs used in the mechanical tests are presented. Tensile tests performed revealed that TMJ discs presented different behaviors for anteroposterior and mediolateral directions (Fig. 7).
Figure 7 Photomicrographs of various regions of the sheep temporomandibular joint (TMJ) disc stained with haematoxylin-eosin. A. Tightly packed collagen fibers with parallel arrangement interspersed by fibroblasts in the central portion of the TMJ disc (bar = 50 μm). B. Haphazardly arranged collagen/fiber bundles in the posterior band of the TMJ disc (bar = 50 μm). C. Chondrocyte-like cells in the anterior band of the TMJ disc (×200, bar = 50 μm). D. Small caliber blood vessels (arrows) in the TMJ disc (×100, bar = 100 μm).

Microphotographies de différentes régions du disque articulaire temporomandibulaire (ATM) de mouton ; coloration à l’hématoxyline-éosine. A. Fibres de collagène denses avec un agencement parallèle intercalées par des fibroblastes dans la partie centrale du disque de l’ATM (×200, la barre = 50 μm). B. Agencement au hasard des faisceaux de fibres de collagène dans la bande postérieure du disque de l’ATM (la barre = 50 μm). C. Cellules chondrocytes-like dans la bande antérieure du disque de ATM (×200, la barre = 50 μm). D. Les petits vaisseaux sanguins de calibre (flèches) dans le disque ATM (la barre = 100 μm).

Indicating parts of the temporomandibular joint that are subject to highest loading, TMJ relation with the external acoustic meatus, foramen ovale and the joint disc position interposing processus condylaris and fossa mandibularis are similar to human TMJ anatomy. TMJ disc morphology is very similar to human TMJ disc. The choice of sheep as an animal model for TMJ studies has been used for several years [16–23]. TMJ disc implants can be an efficacious

Figure 8 Photomicrographs of the central zone (A) and (B) anterior band of sheep temporomandibular joint (TMJ) disc stained with orcinol for detection of elastic fibers (bar = 50 μm). A. Longitudinal elastic fibers follow the wavy structure of collagen bundles. B. Loose mesh elastic fibers distributed between chondrocyte-like cells.

Microphotographies de la zone centrale (A) et (B) antérieure du disque d’une articulation temporomandibulaire (ATM) de mouton colorées par l’orceine pour la détection des fibres élastiques (la barre = 50 μm). A. Les fibres élastiques longitudinales suivent la disposition ondulée des faisceaux de collagène. B. Réseau lâche de fibres élastiques en vrac réparties entre les cellules chondroïdes.
Table 2. Mechanical tensile properties of TMJ discs.

<table>
<thead>
<tr>
<th>Tensile test</th>
<th>Tensile modulus (MPa)</th>
<th>Tensile strength (MPa)</th>
<th>Elongation at break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anteroposterior</td>
<td>3.97 ± 0.73</td>
<td>4.34 ± 1.22</td>
<td>170.92 ± 47.87</td>
</tr>
<tr>
<td>Mediolateral</td>
<td>9.39 ± 1.67</td>
<td>13.21 ± 0.85</td>
<td>195.23 ± 20.44</td>
</tr>
</tbody>
</table>

TMJ: temporomandibular joint. Tensile modulus (E), tensile strength and elongation at break are reported as mean value ± standard deviation.

Figure 9. Tensile mechanical performance of temporomandibular joint (TMJ) discs in anteroposterior and mediolateral directions.

Résistances mécaniques à la traction des disques de l’articulation temporomandibulaire (ATM) dans les directions antéropostérieures et médiolatérales.

Figure 10. Mediolateral tensile mechanical performance of temporomandibular joint (TMJ) discs. Tension tests used a strain rate of 0.5 mm/min with an initial distance between grips (L0) of 2 mm. All tests were conducted on a Zwick Z100 strength-testing machine equipped with a 10 kN load cell.

Résistance mécanique médialatérale des disques de l’articulation temporomandibulaire (ATM). Les essais en traction ont utilisé une vitesse de déformation de 0.5 mm/min avec une distance initiale entre les mâts (L0) de 2 mm. Tous les tests ont été effectués sur un Zwick Z100 équipé d’une cellule de charge 10 kN.

Figure 11. Anteroposterior tensile mechanical performance of temporomandibular joint (TMJ) discs. Tension tests used a strain rate of 0.5 mm/min with an initial distance between grips (L0) of 2 mm. All tests were conducted on a Zwick Z100 strength-testing machine equipped with a 10 kN load cell.

Résistance mécanique antéropostérieure en traction des disques articulation temporomandibulaire (ATM). Les essais en traction ont utilisé une vitesse de déformation de 0.5 mm/min avec une distance initiale entre les mâts (L0) de 2 mm. Tous les tests ont été effectués sur un Zwick Z100 équipé d’une cellule de charge 10 kN.

Complement to bioengineered joint reconstruction and animal models may offer the possibility to conduct informative preclinical studies. One of the most important problems to create an effective TIL is to replicate the biomechanics characteristics of the native disc. Therefore, information on the biomechanical properties of the substitute material is indispensable for further investigation in TMJ disc tissue engineering. During mandibular movements the TMJ disc is subject to a multitude of different loading regimes. TMJ disc behaves as a viscoelastic structure acting as a stress absorber and a stress distributor [24,27]. Elastic fibers play an important role providing the disc with the necessary viscoelastic structure. During every type of loading, the disc undergoes a deformation, while internal forces are produced within the tissue [24]. The internal forces are quantified by the amount of stress, which is defined as force per unit area in Pa (1 kPa = 1 N/m²). There are only two studies available on bovine TMJ disc in which tensile and compressive modulus have been compared using the same experimental protocol and material [28,29]. In these studies tensile modulus ranged between 22 and 26 MPa, and compressive...
Sheep and temporomandibular joint


Novel approach for 3D virtual model of TMJ disc morphology: Preliminary results

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Introduction: Regenerative medicine is an immense field with extreme and challenging obstacles. The first challenge is to understand the morphological, histological, biochemical and biomechanical characteristics of the structure to reproduce. This multi-task and multidisciplinary approach is essential to determine the success of regenerative medicine. The authors present a new method to reproduce 3D morphology of anatomical structures with sheep TMJ disc as an example.

Objective: The main objective of the authors was to reproduce a 3D virtual model of six sheep TMJ discs.

Methods: A medical surgeon performed surgical dissection of TMJ disc in fresh sheep cadaver with microscope. The second step was related to remove all disc muscular attachments to obtain a clean cartilage disc. The disc was submerged in a solution to maintain the correct morphology. With a white light 3D scanning system and appropriate software we reproduce the morphology of six sheep TMJ disc.

Conclusion: 3D virtual model of TMJ disc were successfully reproduced using a white light 3D scanning system. This technique have economic and time related advantages. For precision and detailed results we need to conduct more studies.

The use of platysma for orbital reanimation

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Lagophthalmos is the result of the paralysis of orbicularis oculi in facial palsy, which can lead to ulceration of the eye and blindness. Unlike static procedures, muscle transfer surgery allows the restoration of involuntary blink reflexes of the orbicularis oculi. As the platysma is like the orbicularis oculi in thickness, it is hypothesised the platysma would be an ideal candidate for muscle transfer surgery. The aim was to determine the neurovasculature of the platysma for reanimation of the orbit. Results were consistent between one platysma from cadaver A (A) and one platysma from cadaver B (B). The numbers of arterial branches identified in each platysma were as follows: facial artery A-7, B-2; submental artery A-3, B-1, and occipital artery A-1, B-1. The venous drainage branches identified were: anterior jugular vein A-1, B-2; external jugular vein A-2, B-1; and facial vein A-2, B-1. All nerves were identified as branches of the facial nerve: A-7, B-7. From the dissection it was found the posterosuperior lateral portion of platysma was more vascular rich, containing branches from the facial artery and facial vein, as well as the facial nerve. This portion of the muscle therefore has potential for use as a muscle transfer flap in surgery, as the rich neurovascular allows adequate rewiring for dynamic restoration. In particular, the muscle transfer flaps would allow the application of the trouser graft procedure, for reanimation of the whole orbit.

Anatomic variation in the pterygoplatine angle of the maxillary sinus—a cone beam CT study

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A retrospective cone beam CT study was performed in 50 patients to evaluate the possibilities of variation in the anatomical sinus of the orbital process of the palatine bone (OPPB). In that sinus were found posterior ethmoid air cells, such as Siew’s and Onodi’s cells, the maxillary recess of the sphenoidal sinus (MRSMS) and the sphenoidal recess of the maxillary sinus (SMRS). The middle and, respectively, superior nasal meatus were also projecting lateral recesses in the postero-supero-medial angle of the maxillary sinus, these being previously unknown variants. In a single case the atrophic left maxillary bone was bicornal, with an anterior hypoplastic maxillary sinus and the posterior chamber being a huge downshifted posterior ethmoid air cell. We determined which of these variant paraconatomizations produced a maxillary bulla (MB) within the maxillary sinus. The most frequently occurring pneumatizations were Siew’s cells (58 % on the right side, 64 % on the left) and MRSMS (20 % on the right side, 22 % on the left), and these were those producing, but not
Bioengineered Temporomandibular Joint Disk Implants: Study Protocol for a Two-Phase Exploratory Randomized Preclinical Pilot Trial in 18 Black Merino Sheep (TEMPOJIMS)

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Abstract

Background: Preclinical trials are essential to test efficacious options to substitute the temporomandibular joint (TMJ) disk. The contemporary absence of an ideal treatment for patients with severe TMJ disorders can be related to difficulties concerning the appropriate study design to conduct preclinical trials in the TMJ field. These difficulties can be associated with the use of heterogeneous animal models, the use of the contralateral TMJ as control, the absence of rigorous randomized controlled preclinical trials with blinded outcomes assessors, and difficulties involving multidisciplinary teams.

Objective: This study aims to develop a new, reproducible, and effective study design for preclinical research in the TMJ domain, obtaining rigorous data related to (1) identify the impact of bilateral discectomy in black Merino sheep, (2) identify the impact of bilateral discectomy in black Merino sheep, and (3) identify the impact of three different bioengineering TMJ discs in black Merino sheep.

Methods: A two-phase exploratory randomized controlled preclinical trial with blinded outcomes is proposed. In the first phase, nine sheep are randomized into three different surgical bilateral procedures: bilateral discectomy, bilateral discectomy, and sham surgery. In the second phase, nine sheep are randomized to bilaterally test three different TMJ bioengineering disk implants. The primary outcome is the histological gradation of TMJ. Secondary outcomes are imaging changes, absolute masticatory time, ruminant time per cycle, ruminant kinetics, ruminant area, and sheep weight.
RESULTS: Previous preclinical studies in this field have used the contralateral unoperated side as a control, different animal models ranging from mice to a canine model, with nonrandomized, unblinded and uncontrolled study designs and limited outcomes measures. The main goal of this exploratory preclinical protocol is to set a new standard for future preclinical trials in oromaxillofacial surgery, particularly in the TMJ field, by proposing a rigorous design in black Merino sheep. The authors also intend to test the feasibility of pilot outcomes. The authors expect to increase the quality of further studies in this field and to progress in future treatment options for patients undergoing surgery for TMJ disk replacement.

CONCLUSIONS: The study has commenced, but it is too early to provide results or conclusions.

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KEYWORDS
Temporal mandibular joint disorders (TMJ); Temporomandibular joint bioengineered disk implants; Temporomandibular randomized preclinical trial protocol

Introduction
The temporomandibular joint (TMJ) is the most frequently affected joint in the human body. The TMJ opens and closes 1500 to 2000 times daily and is essential for everyday functions of the mouth, such as mastication, speech, deglutition, yawning, and snoring, involving special mandatory synergy of both articular sides [1]. The TMJ disk is an essential component in the normal TMJ and has the following functions: (1) it distributes the intra-articular load, (2) it stabilizes the joints during translation, and (3) it decreases the wear of the articular surface [2,3]. The majority of TMJ disorders (TMD) are successfully treated with reversible, conservative, and low-tech treatments such as education and counseling, therapeutic exercises, splint therapy, and pharmacotherapy [4,5].

When the TMJ disk is displaced, malformed, or damaged, it may induce serious internal pathologic processes and/or osteoarthritis [6,7]. Currently, patients suffering from severe TMD have limited validated treatment options. Most surgical approaches, such as TMJ discectomy, do not restore the structural or biological properties of the articulation and disk. This procedure may not be ideal because the TMJ is left without an important functional structure. A variety of interpositional materials have been used to replace the removed disks, including synthetic materials manufactured from silicone, Teflon, polytetrafluoroethylene, and biological interpositional grafts taken from different anatomical sites [8-11]. These interpositional materials do not take into consideration the anatomy and biochemical and biomechanical characteristics of the TMJ native disk [12], and some of them have been associated with serious complications for the patients [8,13,14]. In the late 1980s, Proplast/Teflon TMJ (synthetic interpositional implant) were found to be harmful in many patients. The breakdown of the material, probably caused by TMJ high biomechanical forces, lead to fragmented particles that resulted in an immune foreign body response that caused problems ranging from severe cutaneous inflammatory reaction in the preauricular and cheek areas [15] to severe degenerative joint disease with perforation into the middle cranial fossa [16,17]. The result was a dramatic clinical spectrum of failures for these implants [19]. In December 1991, the US Food and Drug Administration’s Bulletin recommended immediate removal of all previous TMJ Proplast/Teflon implants because of the mechanical failures, many resulting in progressive bone degeneration [18]. In a 1992 workshop, the American Academy of Oral and Maxillofacial Surgery instructed the discontinuation of Proplast/Teflon [18].

The absence of efficacious options to substitute the TMJ disk can be related to difficulties in the translation of animal evidence to the clinical practice in humans. These limitations are likely related to:
1. the use of heterogeneous animal models with conflicting results, possibly due to variable anatomy and intra-articular loading between species [19,20];
2. the use of the contralateral TMJ as control, which may be associated with contralateral overloading [21];
3. the biomaterials used to replace the disk do not account for the morphologic and biomechanical characteristics of the native disk;
4. absence of randomized controlled trials with binding of outcomes’ assessors; and
5. lack of multidisciplinary teams involved in the project.

Preclinical research should promote the effective translation of knowledge into practice. The previously mentioned aspects can limit the effective translation of quality scientific knowledge into clinical practice and may prevent potential issues from patients, clinicians, and scientific progress.

The contemporary absence of successful options to substitute the TMJ disk is still a major issue for public health. Little has changed in the past decade regarding study designs for TMJ investigation, and the treatment for patients with severe TMJ remains controversial. The main objective of the Temporomandibular Joint Interpositional Material Study (TEMPOJIMS) is to develop a new, reproducible, and effective study design for preclinical research in the TMJ field. The second goal is to progress in bioengineering and regenerative medicine evaluating the benefits of a TMJ bioengineering implant to substitute the damaged native TMJ disk. This preclinical exploratory study is divided into two phases. Phase 1 of this study is a blinded randomized preclinical trial, designed to investigate if the TMJ undergoes important injury in bilateral disectomy, bilateral discectomy, and sham surgery. Phase 2 intentions are to evaluate the safety and efficacy of three different TMJ bioengineering implants using the same rigorous method of phase 1.
Methods

Study Design

The TEMPOJOEMS is a two-phase exploratory randomized controlled preclinical trial planned to gather preliminary information to (1) evaluate a new study design for TMJ investigation; (2) evaluate the black Merino sheep animal model for TMJ investigation; (3) evaluate TMJ behavior under bilateral surgical intervention (disectomy and discectomy) using a histologic primary outcome (microscopic scoring of destructive changes in TMJ) using a modified Mankin scoring system [22]; secondary imaging outcome (imaging scoring of TMJ); (4) testing the applicability of pilot secondary outcomes predominantly for ruminant kinetics; and (5) obtain a baseline for interpretation of TMJ disk bioengineering implants results. Phase II is aimed to test safety and efficacy of three different bilateral TMJ bioengineering disk implants (Figure 1). Outcome evaluators and analysts are blinded for surgical assessments.

Figure 1. Study design.

Animal Model

A variety of strains/breeds of sheep have been used in TMJ investigations. To decrease biological variability, the authors recommended black Merino sheep as the animal model to conduct the study [20]. As recommended, the authors proposed to use "sheep skeletally mature" at ≥2 years of age [23]. The inclusion criteria are certified black Merino sheep, adult (age 2-5 years), female, and in good health condition (veterinary check-up is performed on all animals). Regarding the animal ethical considerations, the study design was approved by the Portuguese National Authority for Animal Health registered with number 026618. The study design and organization respect the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Baseline and Follow-Up Evaluation

The baseline and follow-up evaluations are outlined at particular time points (Figure 2). Pilot secondary outcomes and weight are measured at days 11, 10, and 9 before surgery (details on secondary outcomes are reported in outcomes measures). Transportation to surgical facilities is performed 5 days before surgery to avoid animal stress and allow familiarization to the temporary facilities. Head computerized tomography (CT) scan is performed on the day of surgery taking advantage of preanesthesia sedation. Ten days after surgery, animals are transported to TEMPOJOEMS main facilities. Days 19, 20, and 21 after surgery, the follow-up secondary outcomes start to be recorded every 30 days for 6 months (Figure 2). At the end, animals are sacrificed and a new CT scan is performed to measure the imaging outcome and to begin the histologic preparation.

Major institutions involved in this study are (1) Lisbon Faculty of Medicine for study design, coordination, and statistical analysis; (2) Interdisciplinary Centre of Research in Animal Health in Faculty of Veterinary Medicine for histological preparation and veterinary support of all animals; (3) Centre for Rapid and Sustainable Product Development for bioengineered disk implants (disk I and II); (4) Bioengineering, Surgery, Chemical Engineering, Mechanical Engineering and Materials Science, University of Pittsburgh, for bioengineered disk implants (disk III); (5) Department of Oral and Maxillofacial-Head and Neck Surgery, University Hospital Infanta Cristina, Badajoz, Spain, for surgical support; (6) Institute of Bone and Joint Research-Northern Sydney Local Health District-Sydney Medical School Northern University, Sydney, Australia, for histological analysis; and (7) Radiology Department of Santa Maria Hospital, Lisbon, Portugal, for imaging analysis.
Randomization, Allocation, and Blinding
The randomization is performed by a statistical group not involved in the outcome assessments, managed by Lisbon Faculty of Medicine. Allocation to each randomized group is performed preoperatively by sealed envelope and separately for phase 1 and phase 2 of the study. The surgical team is not blinded to treatment allocation given the type of intervention; however, surgical team members are not involved in outcome assessments. All outcome evaluators are blinded to intervention.

In phase 1, 10 sheep are allocated to the intervention group: sham surgery group (n=3), discectomy group (n=3), discectomy group (n=3), and backup group (n=1). The backup sheep is planned to be used if death occurs due to anesthesia or another complication not related to the surgical intervention. In phase 2, 10 sheep are randomly assigned to disk I group (n=3), disk II group (n=3), disk III group (n=3), and backup group (n=1) (Figure 1).

Intervention Phase
Anesthesia Protocol
Fasting and water restriction are required 24 hours before surgery. Sedation is performed with diazepam (0.5 mg/kg iv), followed by anesthesia induction with ketamine (5 mg/kg iv). Oral intubation is performed and anesthesia is maintained with isoflurane (1.5% to 2%). To assure minimal analgesia, meloxicam (0.5 mg/kg iv, b.i.d.) is administered on surgery day and during 4 days postoperatively. Antibiotic prophylaxis with amoxicillin and clavulanic acid are used for 5 days.

Surgical Intervention Protocol for Phases 1 and 2

Phase 1
Bilateral discectomy (n=3): under general anesthesia, the surgical team perform a preauricular skin incision and a blunt dissection of the soft tissue covering the joint. The joint area is disclosed and the articular capsule is incised. The disk and its attachments are identified. The medial, anterior, posterior, and lateral disk attachments are detached and discectomy is performed. The wound is closed in layers.

Bilateral discectomy (n=3): under general anesthesia, the surgical team perform a preauricular skin incision and a blunt dissection of the soft tissue covering the joint. The joint area is disclosed and the articular capsule is incised. The disk and its attachments are identified. The lateral and posterior disk attachments are detached and sutured with poly-p-dioxanone (PDS) 3/0. The wound is closed in layers.

Sham surgery (n=3): under general anesthesia, the surgical team will perform a preauricular skin incision and a blunt dissection of the soft tissue covering the joint. The capsule is not incised. The wound is closed in layers.

Phase 2
Disk I (n=3): under general anesthesia, the surgical team perform a preauricular skin incision and a blunt dissection of the soft tissue covering the joint. The joint area is disclosed and the articular capsule is incised. The disk and its attachments are identified. The medial, anterior, posterior, and lateral disk attachments are detached and discectomy is performed. The disk I is introduced into the articular space and sutured in the lateral attachments. The wound is closed in layers. Disk I will be an alternative biomaterial and for intellectual reasons cannot be revealed in this paper.

Disk II (n=3): under general anesthesia, the surgical team perform a preauricular skin incision and a blunt dissection of the soft tissue covering the joint. The joint area is disclosed and the articular capsule is incised. The disk and its attachments are identified. The medial, anterior, posterior, and lateral disk attachments are detached and discectomy is performed. The disk II is introduced into the articular space and sutured in the lateral attachments. The wound is closed in layers. Disk II will be a porous poly(glycerol sebacate) (PGS) scaffold reinforced with polycaprolactone (PCL).

Disk III (n=3): under general anesthesia, the surgical team perform a preauricular skin incision and a blunt dissection of the soft tissue covering the joint. The joint area is disclosed and the articular capsule is incised. The disk and its attachments are identified. The medial, anterior, posterior, and lateral disk attachments are detached and discectomy is performed.
Disk III is introduced into the articular space and secured in the lateral attachment. The wound is closed in layers. Disk III will be a porous PGS scaffold prepared by a modified salt fusion method. Briefly, ground salt particles (150 mg) with a size range of 25 to 52 μm will be placed into a 3-D printed mold. The mold will be transferred to an incubator at 37°C and 90% relative humidity for 1 hour. The fused templates of salt particles will dry in a vacuum oven at 90°C and 100 millitorr (mTorr) overnight, removing salt cake carefully from the mold before further processing. Fresh made PGS dissolved in tetrahydrofuran (THF; 20 wt%, 380 μL, salt/PGS=2:1) added to the salt cake, and the THF is allowed to evaporate completely in a fume hood for 30 minutes. The salt cake is transferred to a vacuum oven and cured at 150°C and 100 mTorr for 24 hours. The resultant PGS-impregnated salt templates are soaked in deionized water for 4 hours, and then replaced with water for 4 hours, with water exchange every 4 hours during the first 12 hours. After the 12-hour water bath, scaffolds are transferred to deionized water for another 24 hours with water exchange every 8 hours. The resultant scaffolds are frozen down at -80°C and then the lyophilization process is applied.

Ten days for recovery is contemplated for wound care and postoperative medication (see Figure 2).

**Outcome Measures**

The primary outcome is the microscopic scoring of destructive changes in the TMJ using a modified Mankin scoring system [22]. Secondary outcomes are imaging scoring of TMJ destructive changes, absolute masticatory time, ruminant time per cycle, ruminant kinetics, ruminant area, and sheep body weight. Primary and secondary outcome parameters are outlined in more detail in Figure 3.

**Primary Outcome**

The goal is to evaluate histologic gradation of TMJ destructive changes. The time point is 6 months following surgical intervention.

Six months after surgery, the TMJ is removed using a necropsy bone oscillatory saw according to the following anatomic references: cranial (cranial aspect of condylar process in the union region of the zygomatic process), caudal (external to acoustic meatus), dorsal (reference is established to the squamous temporal bone), and ventral (reference is fixed 2 cm below the acoustic meatus in the zone of stylohyoid angle). The joints are fixed in 10% buffered formalin for 24 hours and stored in 70% ethanol. Decalcification is obtained by immersion in 10% formic acid in 5% formalin for up to 20 days, after which the articulations are cut sagittally through the whole condyle. After decalcifying, TMJ articulations are immersed in three graded methyl salicylate/paraffin mixtures and cut sagittally through the lateral into the central part of the TMJ. Histological sections are sent to Sydney Institute of Bone and Joint Research for histological scoring using a modified Mankin scoring system [22]. This assessment is performed and classified independent by two histologists who will be blinded to intervention. A third histologist will act as arbiter in case of disparity.
Secondary Outcomes

The features evaluated are imaging analysis, absolute mastictatory time, ruminant time per cycle, ruminant kinematics, ruminant area, and sheep weight (see Multimedia Appendices 1 and 2). Time point is every month following surgical intervention for a total of 6 months.

To measure secondary outcomes, a specific cage (see Figure 4) was built with a frontal window and a feeder.

Imaging analysis: preoperative CT is performed on all sheep. After animal sacrifice, TMJ blocks are scanned by CT and imaging evaluation is performed using the criteria and score described in Table 1.

### Table 1. TEMPOJMS imaging evaluation criteria.

<table>
<thead>
<tr>
<th>Items</th>
<th>Criteria</th>
<th>0 (no change)</th>
<th>1 (mild change)</th>
<th>2 (moderate change)</th>
<th>3 (severe change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shape</td>
<td>Change of joint form</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condyle erosion</td>
<td>Concavity in cortical</td>
<td>May include reformed joint</td>
<td>Small changes; this change may include ≤2 osteophytes</td>
<td>Moderate changes; multiple osteophytes</td>
<td>Severe changes and overgrowth, marginal proliferation</td>
</tr>
<tr>
<td>Temporal erosion</td>
<td>Concavity in cortical</td>
<td>This stage includes normal joint with no signs of condyle erosion</td>
<td>Erosion in one-third of joint surface</td>
<td>Erosion in two-thirds of joint surface</td>
<td>Erosion over all joint surface</td>
</tr>
<tr>
<td>Condyle sclerosis</td>
<td>Cortical thickening of condyle</td>
<td>This stage includes normal joint with no signs of condyle sclerosis</td>
<td>Sclerosis in one-third of joint surface</td>
<td>Sclerosis in two-thirds of joint surface</td>
<td>Sclerosis over all joint surface</td>
</tr>
<tr>
<td>Temporal sclerosis</td>
<td>Cortical thickening of temporal fossa</td>
<td>This stage includes normal joint with no signs of temporal sclerosis</td>
<td>Sclerosis in one-third of joint surface</td>
<td>Sclerosis in two-thirds of joint surface</td>
<td>Sclerosis over all joint surface</td>
</tr>
<tr>
<td>Condyle marrow</td>
<td>Change of underlying trabecular bone</td>
<td>This stage includes normal joint with no change of condyle trabecular bone</td>
<td>Sclerosis in less than half of trabecular bone</td>
<td>Sclerosis in half of trabecular bone</td>
<td>Sclerosis in all trabecular bone</td>
</tr>
<tr>
<td>Temporal marrow</td>
<td>Change of underlying trabecular bone</td>
<td>This stage includes normal joint with no change of temporal trabecular bone</td>
<td>Sclerosis in less than half of trabecular bone</td>
<td>Sclerosis in half of trabecular bone</td>
<td>Sclerosis in all trabecular bone</td>
</tr>
<tr>
<td>Calcification</td>
<td>Development of calcification across joint space</td>
<td>No calcification across joint space</td>
<td>Calcification in one-third of joint surface</td>
<td>Calcification in two-thirds of joint surface</td>
<td>Bony fusion across joint space</td>
</tr>
<tr>
<td>Global appreciation</td>
<td>Normal joint</td>
<td>In general, mild changes</td>
<td>In general, moderate changes</td>
<td>In general, severe changes</td>
<td></td>
</tr>
</tbody>
</table>

This assessment is performed and classified independently by two experienced radiologists who will be blinded to intervention. A third radiologist will act as arbiter in case of disparity.

Absolute mastictatory time: respecting the flowchart (Figure 2), at 9:00 am the animals are placed in individual cages. A dose of 150 grams of dry pellets (Rico Gado A3) are introduced in the feeder and the time until they eat all the pellets is measured with a chronometer (see Multimedia Appendix 1).

Ruminant time per cycle: respecting the timetable (Figure 2), we record 15 ruminatory cycles approximately 4 hours after 150 gram feeding. We use a Canon 2 video camera and images with 25 frames per second. Then, the number of frames per cycle are divided by 25 to obtain time in seconds per cycle (see Multimedia Appendix 2).

Ruminant kinetics: we use the software Foundry Nuke (2D tracking) to perform the ruminatory tracking and to obtain the ruminatory cycle average. With the software After Effects, we convert the 2D tracking into a geometric form (see Multimedia Appendix 2).

Ruminant area: we determine the average of 15 cycles and create a geometric form. Using the software Image J, we perform a quantitative measure in pixels of the ruminant area average.

Weight: according to the timetable, after eating 150 grams of dry pellets the sheep are weighed (see Multimedia Appendix 1).

All assessments are performed by researchers who are blinded to surgical intervention.

http://www.researchprotocols.org/2017/1/e17/
Statistical Analyses
All statistical analyses will be performed using the SPSS version 22 (IBM Corp, Armonk, NY, USA). A cross-sectional analysis will be performed to explore the outcome variables in the three levels of the independent variable before and after the randomized treatment group assignment. The cross-sectional analyses, one-way analysis of variance (ANOVA) will be performed, after testing all the assumptions. For longitudinal analysis, one-way ANOVA with repeated measures will be performed taking as within-subjects effects observations after surgery (months 1 to 6). Fisher least significant difference will be performed as post hoc tests to check for significant differences in the different treatments.

Reporting of Adverse Events
Adverse events related to the study will be considered, including (1) anesthesia events: idiopathic death, pneumothorax, other complications related to anesthesia; (2) surgical technique: massive bleeding, condylar fracture, other complications related to surgical technique; and (3) postoperative events: TMJ infection, joint effusion, decreased appetite, facial paralysis, decreased rumination, decreased weight.

Discussion
This study investigates the effects and adverse effects of (1) bilateral discectomy, (2) bilateral discoscopy, and (3) bioengineered disk implants. Although this preclinical study will primarily serve as a pilot study, we expect to gain a better understanding of the morphologic and histologic changes in TMJ and implications in masticatory kinetics.

So far, results on discectomy are conflicting, Previous preclinical studies in this field [24-33] have used the contralateral unoperated side as a control and different animal models ranging from mice to a canine model. Using the contralateral side as a control can be inappropriate considering contralateral overload influence. Theoretically, we expect to reduce this bias using a bilateral approach. Animal variability in the different studies is a warning about the importance of using the same animal model in further studies concerning TMJ implant investigations. Therefore, our group performed a previous study considering black Merino sheep as a promissory animal model for studies regarding TMJ disk implants investigation, TMJ prosthesis, and TMJ osteoarthritis model. To increase the quality of TEMPOJMS the authors will use a sham surgery control group.

We expect to obtain valuable information related to the phase 1 discoscopy group regarding if the surgical approach promotes intra-articular damage. This can improve future conclusions about attributing possible damage to the intervention itself instead of the TMJ implant. This question is important considering that a surgical approach to place TMJ implants in phase 2 will be required. Again, using a bilateral intervention could reduce a possible bias.

Most preclinical studies have focused on gross morphological/histological assessments and were not designed
to characterize the fundamental altered joint movement (kinetics) or functional consequences. In this study, we include pilot secondary outcomes to evaluate changes in ruminant kinetics. We expect to correlate the primary with the secondary outcomes to understand if they can be used in future TMJ studies. It may be interesting to understand several items:

1. Are there differences regarding masticatory time in the disk groups versus disectomy and discopexy?
2. Is there a correlation between histologic and imaging and kinetics results?
3. Does the ruminant area and geometry change when performing different interventions?
4. Is there a difference regarding ruminant kinetics in the disk groups versus disectomy and discopexy?
5. Do TMJ implants accelerate osteoarthrosis?

Concerning phase 2, the choice of biomaterial is critical. The TMJ implant will be exposed in a mechanical, stressful environment with a limited blood supply that can limit cell migration and in situ regeneration. Testing three different bioengineering discs in vivo and correlating in vitro with in vivo behavior can seriously improve bioengineering strategies to achieve a safe and efficacious TMJ disk implant for humans.

The main strength of this study is the animal model proposed; the conventional and pilot outcomes described; the study design with a randomized, blinded, and placebo control group; and the use of bilateral surgical procedures. Potential limitations of the study include the relatively small sample size. If this study confirms the feasibility of the proposed protocol and initial efficacy of the TMJ disk implants planned, a larger preclinical trial would be warranted to further determine the effectiveness of these discs and promote translation of animal evidence to clinical practice in humans.

**Trial Status**

At the time of submission, the surgical interventions of phase 1 were ongoing at Faculdade de Medicina Veterinária de Lisboa and TEMPO/JMS facilities in Portugal.

**Acknowledgments**

This preclinical trial is supported by Faculdade de Medicina Veterinária da Universidade de Lisboa, Instituto Politécnico de Leiria (Centre for Rapid and Sustainable Product Development), Centro Hospitalar de Setúbal, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa. The authors are grateful to Joaquim Ferreira from Lisbon Faculty of Medicine for study design; to Susan Smith from Institute of Health and Joint Research—Northern Sydney Local Health District; Sydney Medical School Northern, Australia, for histological analysis; to Pedro Nunes from Radiology Department of Centro Hospitalar Lisboa Norte; to Miguel Virgilio for kinematics video recording; and to Joaquim Ângelo and Ermelinda Ângelo for animal logistics control. This study was granted by Portuguese Or企业的 Foundation and by Portuguese Foundation for Science and Technology (FCT) through the following projects: UID/Multi/04044/2013 and PTDC/EMS-SIS/7032/2014.

**Authors’ Contributions**

The contributors, with input from the other investigators, conceived this study protocol. JP, RF, NG, AT, NG, and DA developed the protocol and study materials with input from all investigators. NG, AT, and DA participated in the randomization process. LM will conduct the statistical analyses. FM, RG, and SF will participate in the surgical interventions. CB and SC are the coordinators of the veterinary staff and responsible for the animal anesthesia and animal welfare. DC participated in organization support and was study advisor. FM, NA, and MC are dedicated to disk implants 1 and 2. WJ, JE, and GJ are dedicated to disk implant 3. SR will coordinate the imaging evaluations. MP and FB are responsible for processing the histologic samples and preparing sections. LC group will coordinate histologic scoring system. All authors read and approved the final manuscript.

**Conflicts of Interest**

None declared.

**Multimedia Appendix 1**

Outcomes assessments in TEMPO/JMS main facilities, absolute masticatory time and weight.

[MP4 File (MP4 Video), 210MB - resprot_v63e37_app1.mp4]

**Multimedia Appendix 2**

Outcomes assessments in TEMPO/JMS main facilities. After recording 15 ruminant cycles with a Canon 7D Video Camera we used the software Foundry Nuke (2D tracking) to make the ruminant tracking to obtain the ruminant cycle average in each time period.

[MP4 File (MP4 Video), 4MB - resprot_v63e37_app2.mp4]
References


Abbreviations

CT: computed tomography
PCL: polycaprolactone
PDS: polylactide-co-glycolide
PCL-PGA: polylactide-co-glycolide
TEPOJMJS: Temporomandibular Joint Interpositional Material Study
THF: tetrahydrofuran
TMD: temporomandibular joint disorders
TMJ: temporomandibular joint

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Effects of bilateral disectomy and bilateral discectomy on black Merino sheep rumination kinematics: TEMPOJIMS – phase 1 – pilot blinded, randomized preclinical study

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ABSTRACT

Background: The temporomandibular joint (TMJ) disectomy and discectomy (TEMPOJIMS) is an important surgical technique to improve the quality of life of patients with TMJ disorders. The purpose of this study was to assess the effects of bilateral disectomy and discectomy on sheep rumination kinematics.

Methods: A total of 12 healthy sheep were randomly divided into two groups: disectomy (DG) and discectomy (DC). The sheep were monitored for 6 months after surgery. The rumination frequencies and times were recorded and analyzed using statistical software.

Results: The DG group showed a significant increase in rumination frequency and time compared to the DC group. However, no significant differences were observed in the rumination patterns between the groups.

Conclusions: Bilateral disectomy and discectomy significantly improved the rumination kinematics in sheep, which may have implications for the treatment of TMJ disorders in humans.

Keywords: TMJ disorders; disectomy; discectomy; sheep; rumination kinematics.;
Despite the large number of discectomy and discopexy procedures performed annually, the best of our knowledge there have been no randomized, blinded, controlled trials that have investigated, in human or animal, the jaw movement implications of bilateral discoscopy and bilateral discopexy. Small-size, mid-size, and large animal models have been used to investigate the histological effects of unilateral discoscopy (Bierman and Kau, 1999; Dimitriou and Shaw, 2006; Ogi et al., 1999; Sato et al., 2002; Tong and Tideman, 2009), leading to diverse results, from minor degenerative changes to TMJ analysis. This heterogeneity results are probably due to limitations regarding animal choice, study design, and the use of a unilateral approach with the contralateral side as a control, which may have induced bias in available results (Cohen et al., 2014).

As reported in a survey, commissioned by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) (Kilbourn et al., 2005), only 50% of the 271 randomly chosen articles asserted the hypothesis or objective of the study, and the number and characteristics of the animals used (i.e., species, strain, sex, age, weight). Most of the papers surveyed did not report using randomization (87%) or blinding (65%) to reduce bias in animal selection and outcome assessment. Only 70% of the publications that used statistical methods fully described them and presented the results with a measure of precision or variability (Kilbourn et al., 2005). These findings are a cause for concern, and are consistent with reviews of many research areas, including clinical studies, published in recent years (Kilbourn et al., 2009; Sharma et al., 2010; Van der Wees et al., 2010). Furthermore, most of the previous studies have focused on histological and imaging differences, but additional inputs are essential to obtain a clear understanding of TMJ functionality.

In this paper the authors report, for the first time, on a high-quality, preclinical study that evaluates the impacts of bilateral discoscopy and bilateral discopexy on mastication and ramification kinematics in black Merino sheep, in comparison with a sham surgery control group.

The evaluation of mastication and ramification kinematics of the sheep jaw was based on the normal processes used by ruminants to break down particulate dry matter: (1) initial chewing during feeding and (2) further chewing during ramification (Pearce, 1987). The authors discriminated between the two processes and analyzed them separately. To analyze the initial chewing, the authors examined the time taken to eat a dose of dry pellets, naming this outcome absolute masticatory time. With this outcome the authors expected to determine if TMJ surgical interventions could induce significant changes in the initial chewing time. To analyze the ramification chewing phase, a special cage was created and 15 ruminant chewing cycles were recorded with a video camera. Using computer software (2D tracking and image) software, movements in ruminant were analyzed to obtain: (1) ramification time per cycle, (2) ramification kinematics, and (3) ramification area.

The temporo-mandibular joint imaging study (TEMPOJMS) was planned with a rigorous design, in line with the ARRIVE guidelines (Kilbourn et al., 2010). A Randomized, preclinical study with blinded outcomes, was needed in this field in order to increase the quality of further TMJ studies, improve future treatment options for patients undergoing surgery for TMJ disc replacement, and facilitate interpretation of future studies regarding TMJ interosseous muscles using TEMPOJMS design.

2. Materials and methods

TEMPOJMS study was a preclinical study divided into two phases (Angelo et al., 2011). This paper focuses on the kinematic outcomes of phase 1, aiming to understand the impact of TMJ bilateral discoscopy versus TMJ bilateral discopexy, in comparison with a sham surgery control group, on black Merino sheep.
ARTICLE IN PRESS

TEMPOJIMS Phase I

Assessed for eligibility
N=10

Baseline assessments

Randomization

Allocation to
discocent group
n=3
Allocation to
am group
n=3
Allocation to
discocent group
n=3
Backup
group
n=1

Surgical
Intervention
6 months
follow-up and
secondary outcomes assessments

Analysis and primary outcome assessment

Fig. 1. TEMPOJIMS phase I enrollment (Angelo et al., 2017). Baseline assessments: (1) subcutaneous injection of 150 g of dry pellets (Rico-Gah A3) was introduced in the beaver and the litter (as usual during the field) was measured; (2) nomination time per cycle (the authors used a Canon 7D video camera in record 15 nomination cycles approximately 4 h after the 150 g load; (3) registration kinematics and (4) nomination area (the authors used InnoPhoto software (2D tracking) to track the jaw and used Fiji software to convert the 2D tracking into a geometric form).

detached and discocent was performed. The wound was closed in layers with Vicryl 3/0.

Bilateral discocent group (n = 3): during general anesthesia, the surgical team performed a preauricular incision and a blunt dissection of the soft tissue covering the joint. The joint area was dissected and the articular capsule was incised. The disc and its attachments were identified. The lateral and posterior disc attachments were detached and sutured with PDS 3/0. The wound was closed in layers with Vicryl 3/0.

Sham surgery group (n = 3): during general anesthesia, the surgical team performed a preauricular incision and a blunt dissection of the soft tissue covering the joint. The articular capsule was not incised. The wound was closed in layers with Vicryl 3/0.

2.7. Follow-up assessments

Baseline assessment (T0) was performed before surgery on days -11, -10, and -9 (Table 1). Ten days after surgery, animals were transported to TEMPOJIMS facilities. Follow-up recording of outcomes began on days 10, 20, and 21 after surgery (T11) and was repeated every 30 days for 6 months (Fig. 2). T0–T6 were based on the means of the three measurements. The assessments were

Fig. 2. Flow chart of TEMPOJIMS – phase I (Angelo et al., 2017).

performed by two specially trained assessors who were not affiliated with the interventions. All animals had bilateral scars to reduce possible bias.

2.8. Kinematic outcomes

Kinematic outcomes evaluated were: (1) absolute masticatory time; (2) ruminant time per cycle; (3) ruminant kinematics; and (4) ruminant area.

To measure the referred outcomes, a specific cage was built with a frontal window and a feeder. All assessments were performed by researchers blinded to surgical intervention, and were designed to evaluate masticatory time changes and to ruminant kinematics. These outcomes were as follows:

(1) Absolute masticatory time: based on the assessment timetable (Fig. 2), at 9:00 am the 10 sheep were placed in their individual cages. A dose of 250 g of dry pellets (Seco Cado A3) was introduced in the feeder and the time taken to eat all the pellets was measured with a chronometer.

(2) Ruminant time per cycle: based on the assessment timetable (Fig. 2), we recorded 15 ruminant cycles approximately 4 h after the 150 g feed. We used a Canon 7D video camera to record images at 25 frames per second. The number of frames per cycle was then divided by 25 to obtain time in seconds per cycle.

(3) Ruminant kinematics: we used Foundry Node (2D tracking) software to track jaw movements and calculate the average ruminant cycle. Using After Effects software, we converted the 2D tracking into a geometric form.

(4) Ruminant area: we determined an average for 15 cycles, and created a geometric representation. Using the software image, we performed a quantitative measurement, in pixels, of the average ruminant area.

2.9. Statistical analysis

The TEMPOJIMS phase 1 randomized, controlled, preclinical trial used ten black Merino sheep, with a 6-month follow-up. The primary analysis tested the effects of the independent variable (IV) for three experimental conditions: 1 = bilateral disectomy; 2 = bilateral disectomy; 3 = sham surgery, using a series of pre-tests (T0) and post-tests (T1 to T6). The dependent variables (outcome measures) were: the time to eat 150 g of pellets; the ruminant time per cycle; and the ruminant area. These events were measured three times in the pre-tests to promote invariance in the outcome measures before surgical intervention (IV). The secondary tests (post-tests) analyzed the outcomes using three measurements at six time intervals (T1 to T6), at the same place, and hour as the pre-tests (Fig. 2).

All statistical analyses were performed using the Statistical Package for Social Sciences (IBM SPSS, version 22.0). Shapiro-Wilk tests were performed for pre-tests (T0) and post-tests (T1 to T6), showing a normal distribution in all groups for all tests (p > 0.05), except for T4 and T6 ruminant areas for disectomy (Shapiro-Wilk = 0.761 and 0.386; p < 0.05).


Fig. 2. Ruminant cycle kinematics (A) – initial portion (B) – maximum open mouth (C) – maximum lateral movement (D) – end of ruminant cycle.
### Table 1: Baseline descriptive statistics.

<table>
<thead>
<tr>
<th>Sheep ID</th>
<th>Birth date</th>
<th>Baseline Mean of three measures</th>
<th>Ruminant kinematics and average area/day</th>
<th>Ruminant cycle size per cycle</th>
<th>Absorption randomized process</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Weight/Kg</td>
<td>Absolute masticatory time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8288</td>
<td>11/01/11</td>
<td>56.0</td>
<td>85.0</td>
<td>6440</td>
<td>0.74</td>
</tr>
<tr>
<td>9705</td>
<td>02/04/12</td>
<td>70.3</td>
<td>90.7</td>
<td>5252</td>
<td>1.21</td>
</tr>
<tr>
<td>8264</td>
<td>16/07/10</td>
<td>56.3</td>
<td>79.3</td>
<td>7223</td>
<td>0.57</td>
</tr>
<tr>
<td>9982</td>
<td>02/06/12</td>
<td>56.0</td>
<td>76.0</td>
<td>6594</td>
<td>0.94</td>
</tr>
<tr>
<td>9960</td>
<td>30/10/00</td>
<td>57.0</td>
<td>89.7</td>
<td>7794</td>
<td>0.90</td>
</tr>
<tr>
<td>8284</td>
<td>16/02/11</td>
<td>68.0</td>
<td>97.7</td>
<td>6504</td>
<td>0.93</td>
</tr>
<tr>
<td>8267</td>
<td>13/07/10</td>
<td>75.7</td>
<td>71.7</td>
<td>6594</td>
<td>0.73</td>
</tr>
<tr>
<td>9701</td>
<td>07/04/12</td>
<td>63.0</td>
<td>108.7</td>
<td>10356</td>
<td>1.14</td>
</tr>
<tr>
<td>1903</td>
<td>25/12/12</td>
<td>52.0</td>
<td>101.3</td>
<td>6007</td>
<td>0.74</td>
</tr>
</tbody>
</table>

* No significant differences between sheep for the reported characteristics were found at baseline; p < 0.05.
* The absolute masticatory time was measured from 1400 am, when a dose of 150 g of dry pellets (Oro-Gate AD) was introduced in the feeder; until all pellets were eaten.
* Ruminant kinematics refers to the average tracking of 15 rumination cycles and the creation of a geometric form using the software ImageJ.
* A-Cause TV video camera set at 25 frames per second was used to record 15 rumination cycles approximately 4h after the 150 g feed. The number of frames per cycle was divided by 25 to obtain the time in seconds per cycle.

Additionally, Levene statistics were performed to test the homogeneity of variances. Statistically significant results were found at T1, T2, and T5 for rumination area (Levene statistics = 8.59, 6.35, and 7.82; p < 0.05), which led to non-parametric tests being calculated for these times. For the pre-tests and other time groups, variances were homogeneous (p > 0.05), leading to parametric tests. A one-way analysis of variance (ANOVA) (or the non-parametric equivalent Kruskal-Wallis test) was performed for cross-sectional analysis to compare the outcome variables at the three levels of the IV before and after the random treatment group assignment. Fisher LSD and Games-Howell Post-hoc tests were performed for equal variances assumed and not assumed, respectively. For longitudinal analysis, Mauchly’s test of sphericity was non-significant for absolute masticatory time (Mauchly’s W = 0.004; p = 0.589), allowing a parametric one-way ANOVA test with repeated measures, taking as within-subject effects observations before (T0) and after (T1 to T5) surgery for bilateral discectomy, bilateral discovery, and shunt surgery conditions. For rumination area, a Greenhouse-Geisser corrected test was used, due to a Mauchly’s W value of 0.000; p = 0.011.

### Results

Descriptive baseline statistics are presented in Table 1. Four outcomes were analyzed: (1) absolute masticatory time; (2) rumination time per cycle; (3) ruminant kinematics; and (4) rumination area.
TABLE 3

Absolute maximal time for T0 (baseline) to T1–T6 (post-test); description one-tailed ANOVA for T0 and T3–T6, Mood–Kendall–Wallis test for T1 and T2; effect sizes (w²) and observed power (1–β).

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>784.6 ± 100.8</td>
<td>1036.3 ± 6.5</td>
<td>860.3 ± 17.6</td>
<td>793.9 ± 13.2</td>
<td>852.5 ± 11.9</td>
<td>793.3 ± 7.2</td>
<td>247.4 ± 50</td>
</tr>
<tr>
<td>T1</td>
<td>971.1 ± 11.8</td>
<td>1012.3 ± 5.4</td>
<td>867.4 ± 9.2</td>
<td>984.4 ± 15.7</td>
<td>862.4 ± 18.1</td>
<td>96.0 ± 12.1</td>
<td>92.8 ± 76</td>
</tr>
<tr>
<td>T2</td>
<td>963.1 ± 11.0</td>
<td>8935.2 ± 5.5</td>
<td>853.3 ± 18.8</td>
<td>847.6 ± 16.3</td>
<td>694.9 ± 13.7</td>
<td>92.6 ± 11.7</td>
<td>85.6 ± 80.2</td>
</tr>
<tr>
<td>F (w²)</td>
<td>1.88</td>
<td>5.5*</td>
<td>0.85</td>
<td>0.45</td>
<td>0.70</td>
<td>1.30</td>
<td>1.70</td>
</tr>
<tr>
<td>W²</td>
<td>0.109</td>
<td>0.24</td>
<td>0.14</td>
<td>0.23</td>
<td>0.17</td>
<td>0.04</td>
<td>0.37</td>
</tr>
<tr>
<td>1–β</td>
<td>0.27</td>
<td>0.00</td>
<td>0.10</td>
<td>0.14</td>
<td>0.11</td>
<td>0.27</td>
<td>0.24</td>
</tr>
</tbody>
</table>

*p = 0.03; one-tailed test.

1. Absolute maximal time

Cross-sectional analysis: The authors compared the absolute maximal times for the three groups each month, post-surgery (T1 to T6). A one-way ANOVA (or the non-parametric equivalent Mood–Kendall–Wallis test) was performed, showing significant differences between the three groups only in T1 (p = 0.03, one-tailed), effect size of w² = 0.726 (1–β = 0.82, Table 2) due to the higher value for recovery in comparison with sham surgery, as shown using a Games–Howell post-hoc test (p = 0.023).

Throughout the baseline and the remaining follow-up period (T2–T6), no statistically significant differences were found between disectomy, discography, and sham surgery conditions (p > 0.20).

Longitudinal analysis: A one-way ANOVA with repeated measures was performed, taking as within-subject effects months before (T0) and after surgery (T1 to T6) for discography, discography, and sham surgery conditions. Significant effects across time were found for discography (F(6, 12) = 5.61, p = 0.005, w² = 0.739 (1–β = 0.847), but not for discography and sham surgery (F(6, 12) = 2.65 and 1.59, p = 0.07, w² = 0.57) and 0.443 (1–β = 0.635 and 0.40, respectively). Considering the differences in relation to the baseline (Table 4), the within-subject contrasts identified a statistically significant increase only for discography between T0 and T1 (effect size of 0.98, observed power of 0.97) and between T0 and T14 (effect size of 0.85, observed power of 0.74). For discography and sham surgery, despite the effect sizes and considering the low observed powers, the differences in relation to the baseline were not statistically significant. Fig. 4 represents absolute maximal time for the baseline and from T1 to T6.

2. Ruminant time per cycle

Cross-sectional analysis: Ruminant time per cycle rate did not vary across groups both in the pre-test (T0) and in all times for the post-test (p > 0.20), as shown in Table 4.

Longitudinal analysis: A one-way ANOVA with repeated measures was performed, taking as within-subject effects the baseline and the 6 months after surgery for discography, discography, and sham surgery (see Table 5). A significant effect across time was found for discography and sham surgery (F(6, 6) = 6.87 and 4.17, p < 0.018, w²p = 0.773 and 0.673 (1–β = 0.077 and 0.845, respectively) but not for discography (F(6, 6) = 2.70, p = 0.126, w²p = 0.730 (1–β = 0.45).

The comparison of rumination time per cycle rate between the baseline and months after the surgery identified two differences for discography (T5 vs. T10) with an acceptable power (effect size of 0.55). For discography and sham surgery no significant differences were found in relation to baseline. Fig. 5 illustrates the rumination time per cycle rate in the baseline, and from T1 to T6. As can be seen, lower scores were obtained for times T1, T2, and T3, suggesting that a sheep (T1) recovery started at T4.

3. Ruminant kinematics and area

Descriptive results for rumination kinematics and average area of rumination are presented in Fig. 6.

Cross-sectional analysis: Rumination areas only varied across groups in T3 and T4. For T3, the Fisher LSD post-hoc test identified a significant superiority for the discography area compared with the discography area (p = 0.008) (see Table 6).

Longitudinal analysis: A one-way ANOVA with repeated measures, with Greenhouse–Geisser correction, taking as within-subject effects the baseline and the 6 months after surgery (T1 to T6) for discography, discography, and sham surgery, did not show statistically significant differences for the three conditions (p > 0.10). The differences between pre-test and post-test times were also not statistically significant (p > 0.05), with lower power, since (1–β < 0.80, as can be seen in Table 7. Fig. 7 represents rumination area for T0 (pre-test) and T1 to T6 (post-tests), for discography, discography, and sham surgery. The baseline results are similar for the three experimental conditions. After surgery, rumination areas were lower in the discography condition, although the differences were not statistically significant.

3.1. Adverse events

No serious adverse events were reported, apart from one sheep in the discography group that stopped rumination in T1 and T2, but returned to normal function in T3 to T6.

TABLE 4

Comparison of absolute maximal times between baseline and T1–T6 within subjects, effects sizes (w²), and observed power (1–β).

<table>
<thead>
<tr>
<th>Comparison with baseline (T0)</th>
<th>Discography</th>
<th>Discography</th>
<th>Sham surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 vs T0</td>
<td>17.03*</td>
<td>0.90</td>
<td>0.00</td>
</tr>
<tr>
<td>T2 vs T0</td>
<td>1.22</td>
<td>0.38</td>
<td>0.10</td>
</tr>
<tr>
<td>T3 vs T0</td>
<td>1.00</td>
<td>0.00</td>
<td>0.05</td>
</tr>
<tr>
<td>T4 vs T0</td>
<td>1.26*</td>
<td>0.93</td>
<td>0.27</td>
</tr>
<tr>
<td>T5 vs T0</td>
<td>1.50</td>
<td>0.49</td>
<td>0.07</td>
</tr>
<tr>
<td>T6 vs T0</td>
<td>1.50</td>
<td>0.49</td>
<td>0.07</td>
</tr>
</tbody>
</table>

*p < 0.05.
4. Discussion

The main goal of this study was to analyze the effects of different types of surgery on sheep mastication and rumination. The proposed methodology has proven to be feasible and sensitive to the interventions. Homogeneous conditions were obtained in baseline and the animals behaved naturally in front of the camera, guaranteeing the quality of the kinematic assessments (Fig. 3).

The measurement of kinematics was designed to advance understanding of the implications of TMJ surgery on jaw movements. Theoretically, bilateral TMJ surgery may cause jaw movement changes, but these outcomes need to be quantified.

Regarding absolute masticatory time, it was expected that, after bilateral discectomy, the animals would require more time to eat the 150 g of pellets (Ingwall and Goswami, 2000). Accordingly, the discectomy group increased the masticatory time by 28% in T1. This could be related to TMJ pain, leading to a slower food intake. At the end of the study, these animals were able to recover to baseline values (74.67 s) (Fig. 4). As mentioned previously, there is a lack of studies evaluating the effects of interventions on functionality of the TMJ. Thus, it was not possible to compare this masticatory outcome measured with other results. Although there were no statistical differences between masticatory time before and after surgery (i.e., T0 vs T1), the difference was noticeable. After T1, the subsequent recovery to baseline values suggests that sheep presented the ability to adapt to the induced constraints, highlighting the importance that function has over form (Poveda et al., 2007).

Sheep, as with other animals, have the ability to adapt in order to survive, even in the case of TMJ major interventions, where severe dysfunction could lead to disastrous consequences for the animal.

Table 4: Duration time per cycle for T0 to T1- T6: description, one-way ANOVA; effect-sizes (η²), and observed power (1-β).

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration</td>
<td>0.03 ± 0.03</td>
<td>0.21 ± 0.21</td>
<td>0.06 ± 0.06</td>
<td>0.59 ± 0.59</td>
<td>0.09 ± 0.09</td>
<td>0.08 ± 0.08</td>
<td>0.09 ± 0.09</td>
</tr>
<tr>
<td>Discency</td>
<td>0.05 ± 0.10</td>
<td>0.78 ± 0.78</td>
<td>0.08 ± 0.08</td>
<td>0.32 ± 0.32</td>
<td>0.08 ± 0.08</td>
<td>0.08 ± 0.08</td>
<td>0.09 ± 0.09</td>
</tr>
<tr>
<td>Sten</td>
<td>0.86 ± 0.13</td>
<td>0.72 ± 0.72</td>
<td>0.85 ± 0.85</td>
<td>0.66 ± 0.66</td>
<td>0.76 ± 0.76</td>
<td>0.81 ± 0.81</td>
<td>0.88 ± 0.88</td>
</tr>
<tr>
<td>η²</td>
<td>0.02</td>
<td>0.28</td>
<td>0.10</td>
<td>0.07</td>
<td>0.10</td>
<td>0.19</td>
<td>0.92</td>
</tr>
<tr>
<td>1-β</td>
<td>0.35</td>
<td>0.05</td>
<td>0.06</td>
<td>0.11</td>
<td>0.09</td>
<td>0.09</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Appendix: 4. Discussion

The main goal of this study was to analyze the effects of different types of surgery on sheep mastication and rumination. The proposed methodology has proven to be feasible and sensitive to the interventions. Homogeneous conditions were obtained in baseline and the animals behaved naturally in front of the camera, guaranteeing the quality of the kinematic assessments (Fig. 3).

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Sheep, as with other animals, have the ability to adapt in order to survive, even in the case of TMJ major interventions, where severe dysfunction could lead to disastrous consequences for the animal.
Fig. 5. Rumination time per cycle for T0 (baseline) to T1–T6 (post-test) in bilateral discectomy, discopexy, and sham surgery conditions.

Fig. 6. Rumination geometry and average r2s of rumination for T0 (baseline) to T1–T6 (post-test) in bilateral discectomy, discopexy, and sham surgery conditions. *No rumination cycles were detected — rumination r2s was zero.

Table 6
Rumination area for T0 (baseline) to T1–T6 (post-test): describes one-way ANOVA for T0 and T3–T6, and Kruskal–Wallis test for T1 and T2; effect sizes (η²) and observed power (1−β).

<table>
<thead>
<tr>
<th>Time</th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery</td>
<td>waldf</td>
<td>x²df</td>
<td>x²df</td>
<td>x²df</td>
<td>x²df</td>
<td>x²df</td>
<td>x²df</td>
</tr>
<tr>
<td>Discectomy</td>
<td>6953.33</td>
<td>1018.53</td>
<td>5018.23</td>
<td>4470.50</td>
<td>4708.33</td>
<td>4582.23</td>
<td>4003.02</td>
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<tr>
<td>Discopexy</td>
<td>7684.00</td>
<td>2798.41</td>
<td>1094.49</td>
<td>1067.33</td>
<td>1411.05</td>
<td>957.67</td>
<td>2447.16</td>
</tr>
<tr>
<td>Sham</td>
<td>6933.00</td>
<td>904.55</td>
<td>1000.00</td>
<td>158.05</td>
<td>8859.00</td>
<td>1359.00</td>
<td>9385.33</td>
</tr>
<tr>
<td>R2, α</td>
<td>0.93</td>
<td>0.94</td>
<td>0.94</td>
<td>0.94</td>
<td>0.94</td>
<td>0.94</td>
<td>0.94</td>
</tr>
<tr>
<td>η²</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>1−β</td>
<td>0.631</td>
<td>0.156</td>
<td>0.524</td>
<td>0.777</td>
<td>0.625</td>
<td>0.288</td>
<td>0.469</td>
</tr>
</tbody>
</table>

* p < 0.05.

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Table 7

<table>
<thead>
<tr>
<th>Comparison with baseline (T0)</th>
<th>Discorrection</th>
<th>Recovery</th>
<th>Sham surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>χ²</td>
<td>1 - β</td>
</tr>
<tr>
<td>T1 vs T0</td>
<td>0.20</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td>T2 vs T1</td>
<td>0.42</td>
<td>0.17</td>
<td>0.07</td>
</tr>
<tr>
<td>T3 vs T0</td>
<td>2.37</td>
<td>0.54</td>
<td>0.15</td>
</tr>
<tr>
<td>T4 vs T1</td>
<td>0.25</td>
<td>0.11</td>
<td>0.06</td>
</tr>
<tr>
<td>T5 vs T0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.05</td>
</tr>
<tr>
<td>T6 vs T1</td>
<td>0.61</td>
<td>0.23</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* p < 0.05.

Fig. 3. Ruminative area (bias vs. baseline) in T1–T6 (post-test) in discorrection, recovery, and sham surgery conditions.

The authors agree that it would be interesting in the future to analyze this outcome for a longer period.

Regarding the rumination time per cycle, notable results were achieved in the discorrection group where one animal stopped rumination through T1 and T2. This suggests a need for future investigations in this field, to understand why some T1 animals did not continue with rumination. The authors believe that if any prior studies did not observe such a decrease, the sheep were able to adapt and return to normal ruminative behavior. When analyzing Fig. 5, it is noticeable that all groups reduced the rumination time per cycle in T3, without knowing the cause of any event leading to that result. However, in T4–T6 the sheep reassumed expected values. The animals from both discorrection and recovery groups in T3 and T5 needed more time to achieve a ruminatory cycle, suggesting a less effective rumination process.

Regarding rumination area, it is noticeable that a faster ruminant cycle is obtained through a smaller rumination area. Another interesting detail is that in T3 and T4 a normalization of the rumination kinetics was observed for the discorrection group. This outcome suggests that remodeling and adaptation occurs 3–4 months after T4 surgical intervention. Although rumination areas were reduced in the discorrection group after surgery, the differences were not statistically significant.

The evaluation of trajectory and area of rumination was interesting because it was possible to identify a pattern. Each animal showed a favorite side for rumination but switched sides independently of the intervention. Every animal displayed a triangular trajectory, similar to the jaw movements demonstrated in anesthetized rabbits (Tiedeke et al., 1997).

Further research should be able to examine possible associations between these results and histological, imaging, and weight outcomes (Zhao et al., 2010). 5. Conclusions

The authors are not aware of any previous randomized, blinded, preclinical studies of the TMJ domain that follow the ARWE guidelines. Using black Merino sheep, with age and gender selection, a publicly available protocol, sham control group, and a bilateral approach, we intended to minimise possible bias. The bilateral approach also avoided any adverse effects of the unoperated contralateral joint, as have been reported with unilateral procedures (Dimitriou and Slavin, 2008). The proposed baseline outcomes were homogeneous and the sham control group performed effectively.

The first month after intervention seems to be the critical period regarding kinematic changes, with modifications related to absolute masticatory time, rumination time per cycle, and rumination area, both in discorrection and recovery groups. After 1 month, TMJ bilateral discorrection does not seem to have an important kinematic impact in black Merino sheep. TMJ bilateral discorrection does seem to have a significant impact, mostly in T1 and T2, but from T3 to T6 normalization of results is observed.

The authors agree that the rigorous study design, the animal model, and bilateral intervention were the main advantages of this research. The limitations were mostly due to the small sample size, so further research should aim for larger samples. The introduction of kinematic evaluation highlights the importance of kinematics...
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10

Di-Angelo et al. Journal of Cranio-Maxillo-Facial Surgery 2017; 1–16

study in TMJ domain, and represents a new approach for future

Ethical Approval

Portuguese National Authority for Animal Health; registration

number 026618.

Funding

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Competing Interests

None declared.

Patient Consent

Not required.

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161
Preclinical randomized controlled trial of bilateral discectomy versus bilateral discectomy in Black Merino sheep temporomandibular joint: TEMPOJIMs – Phase 1 - histologic, imaging and body weight results

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ABSTRACT

Introduction: The role of temporomandibular joint (TMJ) surgery is not well defined due to a lack of quality randomized controlled clinical trials, comparing different TMJ surgical treatments with medical and placebo interventions. The temporomandibular joint interventional study (TEMPOJIMs) is a rigorous preclinical trial divided in 2 phases. In phase 1 the authors investigated the role of the TMJ disc and in phase 2 the authors evaluated 3 different interpositional materials. The present work of TEMPOJIMs – phase 1, aims to evaluate histopathologic and imaging changes of bilateral discectomy and discovery in Black Merino sheep TMJ using a high-quality trial following the ARRIVE guidelines.

Material and Methods: This randomized, blinded and controlled preclinical trial was conducted in 9 Black Merino sheep to investigate histopathologic (primary outcomes), imaging and body weight (secondary outcomes) changes after bilateral discectomy, discopaxy and sham surgery.

Results: Significant changes were noticed in discectomy group, both in imaging and histopathologic analyses. Body weight changes were most pronounced in the discectomy group in the first 4 months after surgery with recovery to baseline weight 6 months after surgery. Discopaxy induced nonsignificant changes in histopathologic, imaging and body weight analyses.

Conclusion: This study reiterates the importance of developing an effective interpositional material to substitute the TMJ disc and the need to explore the molecular mechanisms that underlie TMJ cartilage degeneration. The study design proposed in TEMPOJIMs represents an important progress towards future rigorous TMJ investigations.

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1. Introduction

In severe temporomandibular disorders (TMD) the standard treatment is mostly surgical (Dimiroulias, 2013). However, the role of temporomandibular joint (TMJ) surgery is not well defined (Dimiroulias, 2005) due to a lack of quality randomized controlled clinical trials, comparing TMJ surgical treatment with medical treatment and placebo (Reston and Turkiewicz, 2003; Souza et al., 2012). TMJ open surgical approaches for severe disorders include mainly discotomy or discopexy, in cases where nothing in the joint is salvageable, a total joint replacement may be necessary (Dimiroulias, 2013). Despite the large number of discotomy procedures performed annually, we are not aware of any rigorously performed, randomized, controlled trials that have been investigated, in human or animal, the effectiveness of discotomy, compared with discopexy, bioengineered interpositional material and sham surgical interventions. Previous studies stated a significant increase in TMJ osteoarthritis (OA), following discotomy with and without replacement of the disc with an interpositional implant (93% and 90%, respectively). These authors presented a reduced incidence of OA (62%) when using discopexy. Still, this technique was associated with frequent relapse, requiring a secondary discotomy (Trumpp and Lyberg, 1995). These outcomes clearly demonstrate the importance of further studies to deepen understand the effects of surgery and progress for future development of interpositional materials.

Most clinical trials use imaging to classify the TMJ degenerative process (Eriksson and Westesson, 2001). Computed tomography (CT) is a valuable tool to evaluate TMJ OA (Cordello et al., 2016) and it is used by most clinical studies to evaluate articular changes (Boman, 1947; Eriksson and Westesson, 1985; Hall, 1985; Kiehn and Desprez, 1986; Silver, 1986; Takaku et al., 1994; Tokuzen et al., 1994). The present long-term follow-up clinical studies presented condylar flattening and sclerosis after discotomy, but these were not associated with TMJ symptoms (Eriksson and Westesson, 1985; Hall, 1985; Silver, 1986; Tokuzen et al., 1994). Oppositely, the Desprez group (1992) suggested an association of articular erosion with pain in the post-operative period (Kiehn and Desprez, 1992). While imaging modalities are key measures in clinical research, preclinical studies provide a unique chance to also obtain histologic pathology to better understand TMJ surgery-induced changes and improve knowledge for interpositional materials research. Previous preclinical studies have evaluated histologic and imaging outcomes using study designs with a potential sources of bias (selection bias, measurement bias, non-randomization, non-blinded outcome assessment, increasing risk of errors in the results of the study, and in further conclusions (Block and Bouvier, 1990; Chosak et al., 1969; Hagan and Almorza, 2012; Laurell et al., 1987; Macfarlane et al., 1992; Ogil, 1996).

The temporomandibular joint interpositional Material Study (TEMPoJMS) was planned with a rigorous pre-published design (Angelo et al., 2017) according to the ARRIVE guidelines (Kilkenny et al., 2010). This first high-quality randomized preclinical study, performed in Black Merino sheep, was required to increase the translational power of further studies and to progress in future treatment options for patients undergoing surgery for TMJ disc replacement. TEMPoJMS is divided into phase 1 and 2. Phase 1 was a randomized, blinded preclinical trial designed to investigate the TMJ imaging (CT), histopathologic, and body weight changes in sheep after bilateral discotomy, discopexy or sham surgery. Phase 2 uses the same design to test different bioengineering scaffolds to repair the TMJ disc in sheep. It is critical that all assessments are performed and classified independently by two professionals, from each area, who are blinded to intervention. In both phases the primary outcome was the histological grading of TMJ pathology. The main goal of the present investigation was to examine the effects of bilateral surgery over the phase 1 outcomes.

2. Material and methods

2.1. Study design

The rationale and protocol for the TEMPoJMS preclinical trial are publicly available (Angelo et al., 2017). An independent data and safety monitoring board unblinded preclinical results. The study was approved by the Portuguese National Authority for Animal Health registered with number 026618. The study design and organization respected the ARRIVE guidelines (Kilkenny et al., 2010).

2.2. Study population and sample

Relevant preclinical TMJ studies have been conducted in sheep (Ishihara and Goss, 1992; Matsura et al., 2006; Miyamoto et al., 1999; Ogil et al., 1996; Takahashi et al., 2007), and to decrease biological variability in TEMPoJMS results, a specific purebred Black Merino sheep strain was used (Angelo DF et al., 2017). In 2016, our group performed an anatomic, biomechanical and histologic study of Black Merino sheep TMJ, highlighting the potential of this animal to conduct preclinical trials in the TMJ domain (Angelo et al., 2016). The following eligibility criteria were used: certified Black Merino sheep, adult (aged between 2 and 5 years), female, and in good health condition (evaluation was performed by veterinarians, also confirming normal dentition).

2.3. Randomization

The randomisation process was performed by a statistical group, not enrolled in the outcome assessments. Ten sheep were randomly allocated to the intervention groups: bilateral sham surgery (n = 3), bilateral discotomy (n = 3), bilateral discopexy (n = 3), and backup group (n = 1). One backup sheep was planned to be used if death occurred due to anaesthesia, or other complication not related to surgical intervention. The allocation to each randomized group was performed preoperatively by sealed envelope.

2.4. Procedures

Ten eligible sheep were selected and baseline body weight was measured at days 11, 10, and 9 before surgery. Transportation to surgical facilities was performed 5 days before surgery to avoid animal stress and allow familiarization to the temporary accommodation. Head CT-scan was performed on the day of surgery taking advantage of pre-anaesthesia sedation (supplementary material doc 1). The surgical team was not blinded to treatment allocation given the type of intervention; however surgical team members were not involved in outcome assessment. Serious adverse events were defined as events that were fatal or life-threatening or persistent disability, or that resulted in death, over 10% weight loss per week, or clinically significant hazard or harm to the animal.

2.5. Intervention phase

2.5.1. Anaesthesia protocol

Fasting and water restriction were required 24h before surgery. Sedation was performed with diazepam (0.5 mg/kg i.v.), followed by anaesthesia induction with ketamine (5 mg/kg i.v.). Oral intuba- tion was performed and anaesthesia was maintained with iso- flurane (1.5 – 2%). To guarantee animal analgesia, metoclo-
(0.5 mg/kg, iv, bid) was administered on the day of surgery up until 4 days post-operatively. Antibiotic prophylaxis with amoxicillin and clavulanic acid (50 mg/kg, iv, bid) was used for 5 days after surgeries.

2.5.2. Surgical intervention

In all animals, the surgical site was shaved, the skin prepared with povidone iodine solution, and isolated with sterile drapes according to standard surgical procedures. With a 10-scalpel blade a 6 cm long pre-auricular skin incision was performed followed by blunt dissection of the soft tissue covering the joint, to expose the articular capsule. Tissue retractors were used to maintain exposure of the surgical field. In the sham group (n = 3) TMJ articular capsule was not incised, and the wound was closed in 3 layers (muscular, subcutaneous and skin) with Vicryl 3/0. In the remaining animals, the joint capsule was incised and the disc and its attachments were identified. In the discocyte group (n = 3), the disc was exposed and using iris scissors the lateral, anterior and posterior attachments were dissected, allowing exposure and transection of the medial attachment and removal of the intact disc. In the discocyte group (n = 3), the lateral and posterior disc attachments were sharply detached using an iris scissors. A 4-6 mm triangular segment of the retrodiscal tissue was removed and then sutured with PDS 3/0. The wound, including joint capsule, was closed in 4 layers (joint capsule, muscular, subcutaneous and skin) with Vicryl 3/0.

2.5.3. Follow-up assessments

Ten days after surgery, animals were transported to TEMPOJMS facilities (Angelo et al., 2017). From the 19th to 21st day after surgery, follow-up secondary outcomes were recorded, which were repeated every 30 days for 6 months (T1 to T6, respectively). Data from T0-T6 were calculated on a mean of the 3-day measurements of each month. Six months after the intervention, immediately after euthanasia, all animals had a second CT scan and the TMJ block was removed to histology.

2.5.4. Outcomes

**Histological analysis:** Intact TMJ was removed using a necropsy bone oscillatory saw according to the following anatomic reference: coronal – cranial aspect of coronoid process in the union region of the zygomatic process; caudal – external to acoustic meatus; dorsal – the squamous temporal bone; and ventral – 2 cm below the margin of the articular disc. Specimens were embedded in paraffin and sectioned through the central part of the TMJ. Four-micron sections were mounted on glass slides, heat dried at 1 h at 65 °C, de-waxed with 3 cycles of 5 min with xylene, and stained with toluidine blue and fast green as previously described (Little et al., 2010). Slides identified by a number code were randomized and shipped to the Raymond Purves Labs for scoring by 2 blinded independent assessors experienced in evaluating sheep joint histopathology (CBL, MMS).

As the normal histomorphology of the TMJ is quite distinct from cartilage in appendicular synovial joints (Murphy et al., 2012) (Fig. 1Aa), a modification of a published scoring system specific for the TMJ was used (Li et al., 2014) (Supplementary material doc2). Briefly, the mandibular and temporal cartilage (structure, cell number, shape and closing, and proteoglycan content and distribution), tidemark, cement line, and subchondral bone (structure, osteocyte number, osteoelastic activation, vascular invasion, and calcified cartilage islands) were separately scored from 0 (normal) to 3 (>70% abnormal). Additionally, the temporal and retrodiscal synovial hyperplasia, fibrosis and inflammatory cell infiltration were also scored from 0 to 3. The summed cartilage (possible maximum score 60 in each condyle), subchondral bone (possible maximum score 15 in each condyle), synovial (possible maximum score 9 in each site), and total (possible maximum score 168) histopathology scores were calculated.

**Imaging analysis:** Imaging evaluation was performed and classified independently by 2 experienced radiologists (RS, LN) who were blinded to the intervention using the outlined criteria (Supplementary material doc3).

**Body mass assessment:** Sheep were weighed immediately after eating 150 g of dry pellets. Body mass assessments were performed by 2 trained evaluators who were not affiliated with the intervention.

2.5.5. Statistical analysis

Statistical analyses were performed using either the Statistical Package for Social Sciences (IBM SPSS, version 22.0) or Statistics Data Analysis (STATA®-corporation version 14.2). The histopathology scores for each parameter in each section of the 2 evaluators were averaged, and following un-blinding the median scores (and score summations) for each treatment group were calculated. Differences between treatments were analyzed by mixed ordinal logistic regression.

A one-way Analysis of Variance (ANOVA) was performed for cross-sectional analysis, to compare the outcome variables in the three levels of the independent variable before and after the random treatment group assignment. For longitudinal analysis, a one-way ANOVA with repeated measures was performed taking as within-subjects effects observations after surgery (T1-T6) for all conditions. Primary analysis rested the effects of the surgical intervention using series pre-test and post-test. Body mass and imaging score were used as dependent variables for degenerative process. Body mass was measured 3 times in the pre-test for supporting invariance concerning the outcome measures before the clinical intervention. The secondary analysis (post-test) assessed the outcomes measuring 3 times, in 6 time-points, one per month at the same place, date and hour as in pre-test (Angelo et al., 2017). To analyse imaging scores, non-parametric tests were performed attending to the sample size and the non-normality of the distribution for most variables in each group, Shapiro–Wilks test ≤ .82, p ≤ .09. Kruskal–Wallis tests were performed for group comparisons, with Bonferroni test for post-hoc multiple comparisons. Partial eta squared (η²p) and Cohen’s d were used for effect size calculations. Cohen’s categories were used to evaluate the magnitude of these effect sizes (small if η²p ≤ 0.5, medium if 0.5 < η²p ≤ 0.8, and large if η²p > 0.8).

3. Results

At baseline, no differences between groups were observed in body mass (sham group: 55.1 ± 2.7 kg, discocyte group: 62.3 ± 6.0 kg, discocyte group: 67.3 ± 10.2, p > .05).

3.1. Histologic results

The morphological appearance of the cartilage and bone in sham operated joints was consistent with that previously described as normal TMJ (Murphy et al., 2013; Li et al., 2015) (Fig. 1Aa). The superficial half of the cartilage depth had a distinct laminar appearance, with sparse flattened cells and limited proteoglycan staining more intense with depth. Beneath this, there was a layer densely populated with cells that had a
mesenchymal appearance, and more intense diffuse matrix proteoglycan staining. The deepest cartilage layer contained mature and/or hypertrophic chondrocytes often surrounded by a proteoglycan rich peri-cellular matrix but little or no inter-territorial proteoglycan. A tidemark separating the upper two layers from the deepest cartilage layers could be observed in some sections, suggesting the lower zone was calcified. An indistinct cement line demarcated the subchondral bone which contained evenly distributed osteoclasts in lacunae, and completely separated the cartilage from sparse marrow spaces lined by osteoblasts in the deeper bone. The synovium in sham-operated TMJ was similar to that in the knee joint in sheep (Smith et al., 2008) with a single lining layer of synoviocytes overlying a loose connective tissue with adipocytes and sparse fibroblasts and collagen.

A variety of pathological changes of varying severity were noted in discoscopy and discoscopy joints (Fig. 1Ab–e). The mildest changes included cartilage thickening, slightly increased matrix and peri-cellular proteoglycan staining, increased cell density, vascular activation and invasion of the sub-chondral bone and calcified cartilage layer, with both the tidemark and cement line being more distinct (Fig. 1Aa). Intermediate cartilage pathology was characterized by surface roughening/fibrillation, loss of typical laminar structure, a marked increase in inter-territorial proteoglycan staining in all layers, cell clumping particularly in the upper zones, and further deep zone vascular invasion (Fig. 1Ac). Further advancement of pathology was evident with erosion and loss of surface zone cartilage, decreased cell density in the mid zone but clumping in all layers, vascular invasion into the mid zone (Fig. 1Ad), and ultimately complete loss of cartilage integrity and marked subchondral bone remodeling (Fig. 1Ae). Accompanying the osteochondral changes, there was synovitis with hyperplasia of surface cells, sub-synovial fibrosis with loss of adipocytes and both peri-vascular and diffuse inflammatory cell infiltration (not shown). Blinded scoring demonstrated a significant increase in total median histopathology score in discoscopy compared with the other groups (Fig. 1B). This was driven by a significant increase

in pathology in cartilage, bone and synovium in disectomy compared with sham-operated joints (Fig. 1C). Discecy surgery displayed some evidence of cartilage and synovial pathology, but this was quite variable and did not reach statistical significance.

3.2. Imaging results

The authors compared the outcomes of all surgery conditions (Table 1). In general, differences were very high ($\chi^2_0$ corresponding to 98.8%, statistical power = .999) for all outcomes, excluding calcification. Considering each outcome, differences were higher for shape, followed by condyle sclerosis, temporal sclerosis, condyle marrow, temporal erosion, condyle erosion, and temporal marrow. The effect size of the differences ranged from 43.4% to 90.8%. Fig. 2A is a representative CT imaging of sham surgery group (Fig. 2A), discecy group (Fig. 2B) and disectomy (Fig. 2C).

Excluding the difference between discecy and sham surgery for temporal erosion ($\alpha = .05$), all the other differences were classified as large ($d > 0.80$). The larger differences were between disectomy and sham surgery ($R^2$ corresponding to 92.9% of degeneration in global appreciation), mainly due to shape ($R^2 = 86.0$), condyle marrow ($R^2 = 83.6$), and condyle sclerosis ($R^2 = 80.1$). Condyle erosion and temporal erosion were the least affected, despite an effect size of $R^2$ of 50.3% and 50.8%, respectively. Temporal sclerosis and temporal erosion showed $R^2$ effect sizes of 71.3% and 62.3%, respectively. Discecy also differed from sham surgery ($R^2$ corresponding to 80.3% of deterioration in global appreciation), although with lower effect sizes in comparison to the differences between disectomy and sham surgery, and only for shape ($R^2 = 80.3$), condyle sclerosis ($R^2 = 76.6$), and condyle marrow ($R^2 = 56.7$)(Table 2 and Fig. 3).

3.3. Body mass results

Cross-sectional analysis. Statistical differences were not found in body mass in the pre-test (T0) and in all times for the post-test ($p > .10$, Table 3).

Fig. 4 can be seen that in the disectomy condition sheep lost weight from month 1 to month 4 and recovered their weight during months 5 and 6 after surgery.

Longitudinal analysis. A one-way ANOVA with repeated measures was performed taking as within-subjects effects months after surgery (T1-T6) for disectomy, discecy, and sham surgery. Statistically significant differences were found, $F(5, 10) = 5.69$, 27.35 and 8.07, $p < .01$, $\chi^2_0 = .82$, .912, and .801, (1 − $\beta$) = .992, 1.00, and .977 for disectomy, discecy, and sham surgery respectively, showing that sheep recovered weight from T1 to T6. The tests of within-subjects contrasts identified that the increase happened from T4 to T5 both in disectomy ($p = .04$), discecy ($p = .01$), and sham surgery ($p = .01$). Despite this increase, only those in the disectomy and sham groups increased their weight over the pre-test in T5 and T6, $t(2) = -5.34$ and $-5.00, p < .04$. In disectomy and sham surgery conditions sheep did not exceed their weight at baseline.

4. Discussion

This is the first temporomandibular preclinical study using a randomized, blinded design respecting ARRIVE guidelines. Using the suitable Black Merino sheep with age and gender selection, sham control group and bilateral approach, the authors aimed to reduce possible bias on results. In humans, TMJ cartilage is different from appendicular synovial joints (Murphy et al., 2013), with the distinctly laminar fibrocartilage with sparse proteoglycan.
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Facsimile

167

Fig. 2. Representative CT sagittal image of TMJ: A- sham group, B- discovery group, C- disectomy group.

Table 2

<table>
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<tr>
<th>Outcomes</th>
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*p ≤ .05, **p ≤ .01, ***p ≤ .001.

reminiscent of meniscus and annulus fibrosus of the intervertebral disc (Melrose et al., 2017; Xiu et al., 2017). In sham-operated joints of Black Merino sheep the TMJ cartilage was histologically very like humans, supporting sheep as a good animal model. Rat (Zhang et al., 2016) and goat (Li et al., 2015) also have a typical TMJ fibrocartilage appearance with the distinct organized layers, while in the mouse (Cohen et al., 2014; Xu et al., 2009) and rabbit (Wu et al., 2015) the laminar structure is less apparent.

The histopathology changes obtained in the sheep TMJ after bilateral disectomy were consistent with other investigations using various species, including mice (Cohen et al., 2014), rats (Zhang et al., 2016), rabbits (Embree et al., 2015) and goats (Li et al., 2015). The authors noticed an increase in proteoglycan and rounded cells and thickening of the cartilage after disectomy. These changes in Black Merino sheep are similar to reports in other animals (e.g. mouse (Cohen et al., 2014; Matias et al., 2016; Xu et al., 2009) and please cite this article in press as: Angelo DF, et al. Preclinical randomized controlled trial of bilateral disectomy versus bilateral discovery in Black Merino sheep temporomandibular joint: TEMPOJMS — Phase 1 - Histologic, imaging and body weight results, Journal of Cranio-Maxillo-Facial Surgery (2018), https://doi.org/10.1016/j.jcmfs.2018.01.005
Table 3
Sheep body mass for T0 (pre-test) to T1-T6 (post-test): Descriptive and one-way ANOVA.

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Fig. 3. Mean scores for TMJ imaging score.

Fig. 4. Sheep weight for T0 (pre-test) to T1 to T6 (post-test) in sham surgery, discectomy and disectomy.

rat (Zhang et al., 2016). They are consistent with a chondroid metaplasia, which is potentially associated with the loss of the disc and increased direct loading in the TMJ cartilage. However, when this first protective phase fails under continued abnormal loading, the joint undergoes degeneration with cell death and cloning, surface erosion, subchondral bone changes and degeneration. This latter phase is well described and similar to that in the sheep knee joint following meniscectomy (Gale et al., 2013; Little et al., 2010). In the discectomy intervention group, the TMJ capsule and the intra-articular environment was preserved. The result, as expected, had less severe histopathologic changes, because the disc remained interposed between the bony surfaces, dissipating loading and...
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Protecting the TMJ cartilage. It is noteworthy that we also found more severe synovitis in discoscopy compared with discoscopy, indicating that the inflammation is not just a reaction to the arthrotomy, but part of the OA process in the joint. The histopathological appearance of the synovitis in the TMJ was the same as that in sheep knee joints with OA (Smith et al., 2008). Still, given the underlying anatomical differences in cartilage, future studies should explore the molecular mechanisms that underlie TMJ OA pathology, to determine their similarities and differences with appendicular joints such as the knee (Young et al., 2005). Such studies could lead to progress in defining the pathophysiology and the management of TMJ degenerative disorders.

Radiographic morphologic changes caused by discoscopy were first reported by Bonnar in 1947, describing "flattening off the articular surface" (Bonnar, 1947). In 1966, similar conclusions were obtained with condylar flattening and sclerosis after unilateral discoscopy, where no osteophytes but severe damage were described (Ekroos and Westesson, 1985). Concomitantly, condylar flattening and sclerosis were the most common radiographic findings in a 3.8 years post discoscopy investigation (Tolvanen et al., 1988). In the present study, these outcomes are reinforced with severe morphological changes observed after bilateral discoscopy. Most statistical differences were noted in shape and condyle sclerosis, corresponding to other authors’ clinical findings (Ekroos and Westesson, 1985; Takaku et al., 2000). While the human condyle is convex and tends to flatten after discoscopy, the sheep condyle is normally flat and tend to a more convex form after discoscopy (Fig. 2c). Nevertheless, condyle sclerosis in all joints after bilateral discoscopy ($R^2 = 80.13$) and change in underlying tracheal bone (condyle bone marrow) were also detected. Cortical breakdown characterized by an initial destructive phase was reported by Agerberg and Lundberg in the first 6 months post-discoscopy (Agerberg and Lundberg, 1971). Some authors suggest that these changes can occur if budding is not controlled during those 6 months (Hall, 1985). Other authors raised the question of whether the lytic condylar process is precipitated by discoscopy or overloading, since the contralateral unoperated joint has similar morphologic changes (Agerberg and Lundberg, 1971; Ekroos and Westesson, 1985; Takaku and Toyoda, 1994; Wilken, 1991). Yailken, in 1979, described the relationship between the condyle and temporal bone 1 year after unilateral discoscopy in Macaca fascicularis (Yailken et al., 1979). Later, Bjornland found fibrous ankylosis 6 months after unilateral discoscopy (Bjornland and Lathlein, 2003). In TEMPOJMS, 6 months after bilateral discoscopy no intra-articular calcification was found, and while fibrous ankylosis cannot be excluded with CT, this was not evident histologically. Significant osteophyte formation is also reported, rarely described in previous studies, which may be due to imaging limitations of radiography and arthrography compared with CT.

To the best of our knowledge, there are no clinical or preclinical studies assessing imaging after discoscopy. Results showed that TMJ open surgery is not innocuous, resulting in mild to moderate changes in global remodeling. The condyle is more affected than the temporal bone and only for shape ($R^2 = 80.31$), condyle sclerosis ($R^2 = 76.68$), and condyle marrow ($R^2 = 56.78$).

In other diseases like rheumatoid arthritis (England et al., 2017), cancer (Lynch et al., 2017), HIV (Mahy et al., 2001) and surgical interventions like gastric sleeve (Casillas et al., 2017), body mass has been used as a valuable outcome to evaluate progress of disease and intervention success. However, for TMJ disorders this outcome has rarely been used. A 4% decrease of body mass in 60% of the animals, 3 months after unilateral discoscopy with condyle and temporal surfaces removal, has been reported (Miyamoto et al., 1995). In a study in mice, after partial discoscopy no significant losses or gains in the body weight of the experimental or control mice were seen (Xu et al., 2009). In this study, after bilateral discoscopy there was 5.2% body mass loss (all occurring in the first month) but with full recovery at 6 months follow-up. In contrast, the discoscopy and sham surgery sheep increased body weight (mostly in T4-T6), finishing the study 8% and 8.2% above the baseline, respectively. It is consistent with the limited TMJ pathology. The evaluation of body mass was also a welfare control measure related to healthy and well fed, respecting the 3 Rs principle (replacement, reduction, or refinement) (Richmond, 2002).

5. Conclusion

This pilot study design demonstrates it is feasible to conduct surgical TMJ preclinical trials in Black Merino sheep. In this study, the authors observed: (1) bilateral discoscopy in a healthy TMJ is not an innocuous intervention, resulting in variable cartilage and synovial pathology along with imaging changes; (2) bilateral discoscopy induced severe TMJ changes detected with both imaging and histopathologic analysis; (3) no fibrous or bony ankylosis was detected over the 6-month period after bilateral discoscopy and discoscopy. And (4) beyond expected cartilage and bone changes, synovitis was shown to be part of the osteoarthritis process, providing a new outcome measure and therapeutic target.

This study has reinforced that: (1) TMJ cartilage is different from appendicular synovial joints, and as such may require unique therapeutic approaches; (2) future investigations are needed to study an effective interpositional material to substitute the TMJ disc and (3) future investigations are needed to explore the molecular mechanisms that underlie TMJ cartilage degeneration.

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Author’s contributions

All the listed authors were involved in drafting the article or revising it critically for important intellectual content, and all the authors approved the final version to be published. Angelo DF, Ferreira, Monje F, Salvador F and Gonzalez R designed the study and carried out the surgical protocol. Angelo DF, Moroço P, Mouta C, Alves N, Virgillo M contributed to data acquisition, Neto L, Sousa R and Caldeira I contributed to imaging blinded scoring. Fábio S, Cavaco S contributed to the animal veterinary support during the investigation. Smith M, Smith S and Little C coordinated the histologic blinded scoring. Monica L contributed to data analysis. Angelo DF, Little C and Moroço P contributed to manuscript preparation.

Conflicts of interest

There are no conflicts of interest between this research and any of the authors herein listed.

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