Master Thesis in Medical Physics

Investigation of the Influence of Different Injectable Biomaterials on the Primary Stability of Cancellous Bone Screws

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Resumen (Español)

Este artículo propone un nuevo método para reforzar las propiedades mecánicas óseas en ciertos casos ortopédicos muy específicos. Se demuestra que un biomaterial no sólido, como un hidrogel conteniendo partículas de hidroxiapatita (HaP), es capaz de proporcionar una estabilidad mecánica relevante a los tornillos óseos desde el momento de su inserción en hueso esponjoso. Mediante micro-tomografía computarizada (µCT) y pruebas mecánicas de tracción hasta la extracción completa de los tornillos, en este estudio se han analizado ciertos parámetros estáticos y dinámicos sobre muestras de hueso esponjoso de fémur bovino. El análisis de imágenes por µCT ha permitido clasificar las muestras en función de parámetros estructurales y de la densidad mineral ósea (BMD). Las pruebas mecánicas han permitido confirmar la hipótesis principal gracias a la diferenciación de las propiedades mecánicas de los biomateriales utilizados. Los resultados mecánicos han mostrado una diferencia significativa (> 200 N para el rango de BMD estudiado) (p-value = 0.0001) en la fuerza de extracción de los tornillos, al normalizarla con los valores BMD (Aumento del 40 % de la fuerza de extracción para BMD = 0.15 g/cm^3) entre un grupo de 12 muestras con un biogel compuesto de ácido hialurónico y HaP (40 %) y las 15 del grupo de control sin biomaterial.

Estudios precedentes habían mostrado que los hidrogeles que contienen HaP son favorables para la formación ósea alrededor de los implantes. Este estudio contribuye al debate sobre la idoneidad de cada biomaterial para cada problema ortopédico, aportando una nueva propiedad mecánica inesperada a estos biomateriales. Se ha demostrado, a través de pruebas mecánicas de extracción de tornillos óseos, que cuando un biomaterial como un gel, basado en ácido hialurónico, contiene suficiente cantidad de HaP (40 %), este sigue siendo inyectable y aporta una estabilidad mecánica primaria al implante.

Abstract (English)

This paper explores the field of cement augmentation of screws in cancellous bone in specific orthopaedic problems. This work demonstrates that a non-setting biomaterial like a hydrogel containing a suspension of Hydroxyapatite particles (HaP) can provide a relevant mechanical stability to cancellous bone screws from the very first moment after the screw insertion. This study was conducted by analysing static and dynamic bone parameters of distal femoral bovine samples. µCT scans and mechanical tests were performed. The µCT analysis helped to classify the samples by structural parameters and cancellous bone mineral density (BMD). While the mechanical tests supported the main hypothesis by differentiating the mechanical properties of the biomaterials used. The results of the mechanical tests showed a significant increase (> 200 N for the range of BMD studied) (p-value = 0.0001) in the pull-out strength of cancellous bone screws normalized with BMD (40 % of pull-out strength increase at BMD = 0.15 g/cm^3) of a group of 12 samples with a bio-gel based in hyaluronic acid with HaP (40 %) when compared to a control group of 15 samples with no-biomaterial.

The current study contributes to the debates on selecting the best biomaterials to each orthopaedic problem by providing a new unexpected property of non-setting hydrogels with HaP, which have already been shown as potentially advantageous for new bone formation. It was shown that a non-setting biomaterial like hyaluronic acid, when loaded with sufficient hydroxyapatite particles (40 %) remained injectable and at the same time provided initial mechanical stabilisation of an implant in bone as measured by a screw pull-out test.

Résumé (Français)

Ce papier évalue le domaine d'utilisation des ciments osseux dans la fixation des vis pour l'os trabéculaire dans certains cas spécifiques de problèmes orthopédiques. Ce travail démontre qu'un biomatériau qui ne durcit pas, comme un hydrogel, et qui contient des particules de Hydroxyapatite (HaP) en suspension, peut apporter une stabilité mécanique aux vis pour l'os trabéculaire dès son insertion. Dans cette étude, des paramètres statiques et dynamiques d'échantillons d'os trabéculaire fémoral bovin ont été analysés. Des scans µCT et des tests mécaniques ont été effectués. Les analyses par imagerie µCT ont permis de classer les échantillons en fonction des paramètres structurels et de la densité minérale osseuse (BMD). Les tests mécaniques ont permis de confirmer l'hypothèse principale grâce à la différentiation des propriétés mécaniques des biomatériaux utilisés. Les résultats mécaniques ont montré une différence significative (> 200 N pour le range de BMD étudié) (p-value = 0.0001) de la force d'extraction des vis trabéculaires normalisée avec BMD (40 % de la force d'extraction augmente à BMD = 0.15 g/cm^3) entre un groupe de 12 échantillons avec un bio-gel composé d'acide hyaluronique avec HaP (40 %) et le groupe de contrôle de 15 échantillons sans aucune biomatériau.

Des études précédentes ont montré que les hydrogels contenant des HaP étaient favorables pour la formation osseuse au tour des implantes. Cette étude contribue à la discussion pour une meilleure sélection des biomatériaux les mieux adaptés aux problèmes orthopédiques en apportant une nouvelle propriété inattendue à ces hydrogels. Il a été montré, au moyen d'un test d'extraction d'une vis osseuse, que lorsqu'un biomatériau non-durcissant est basé sur l'acide hyaluronique et était chargé avec une quantité suffisante de HaP (40%), celui-ci restait injectable et apportait une stabilisation mécanique initiale de l'implant.

To the people who are not afraid of changes.

1. Introduction

In human like in other animals, bone is not absolutely compact. It contains little spaces between bone matrix components and cells. Depending on the size and distribution of these spaces we can differentiate the bone regions in two general classes: compact or cortical bone is the most dense and strong bone formation, containing few spaces and can be found providing protection in the surface of every bone and mechanical support and strength at the diaphysis of long bones; in the other hand, cancellous or trabecular bone is formed by an irregular tridimensional net of thin bone called trabeculae, which leave macrospaces occupied by bone marrow and vessels. Cancellous bone is found in deep regions of many bones and it is usually protected by a cortical shell (Totora and Derrickson 2013). However cancellous bone is weaker than compact bone, it has an important mechanical role as well. This study focuses in the particularities of cancellous bone.

The principal aim of this project is to test if an injectable biomaterial like a hydrogel containing calcium particles is able to increase the primary stability of cancellous bone screws. Conventional injectable calcium phosphate cements (Ooms et al. 2003), (Frayssinet et al. 1998), (ASTM F3087-15 2016) set in situ after injection into the skeleton. The novelty of the biomaterial under consideration in the present study lies in the fact that it does not set and a priori does not become solid. At first sight the concept of a non-solid being loadbearing is counterintuitive. It was hypothesised that the hydrogel, when loaded with sufficient Hydroxyapatite particles could be injectable and at the same time provide initial mechanical stabilisation of an implant in bone.

1.1. Osteopenia and Osteoporosis, the Clinical Problem

Any individual bone constantly undergoes a process of being locally rebuilt known as bone remodelling. Bone remodelling includes bone genesis and resorption and is a normal process necessary to maintain bone mass and strength that reflect the day to day demands on our skeleton. This reshaping process plays also a key role in the calcium equilibrium, stocking Ca in the bones structure or reabsorbing it to the blood flow when needed. In healthy human individuals, the loss of bone is a normal issue that begins after the age of 35 years. "*Bone loss occurs when more old bone is reabsorbed than new bone is created"*. The first level of bone loss is referred to as "osteopenia". Osteopenia is the initial stage of diagnosis of a lower than normal low bone mineral density (BMD). This is measured at the femoral neck and as defined by the World Health Organisation (WHO 2004), as having a T-score -1 to -2.5 (T score is defined as the comparison of bone mineral density of a patient to that of a healthy 30 year old). Osteopenia is considered as a precursor of Osteoporosis, which is a metabolic bone disease that has even lower BMD. However not every case of osteopenia declines to become osteoporosis. Aging is not the only factor producing bone loss. Menopause in women, a lack in vitamin D, many chronic diseases or even a long or permanent incapacity to stand and exercise might produce a significant loss of bone mass and quality making the skeleton more likely to fracture (Van Der Linden et al. 2001), (Lewiecki 2011).

Usually, bone weakening illnesses affect the trabecular bone more than the cortical bone (Singh 2016). Furthermore, after fractures or surgeries the bone healing is more difficult in the cancellous bone (Lu et al. 1998). Eventually this advantage of cortical bone might be beneficial when for therapeutic reasons a bone screw must be placed and fixed in a bone with a thick cortical shell. It is reported (Seebeck et al. 2005) that in bony regions with thin cortices (< 1.5 mm) the screw pull-out strength is dependent on the strength of the underlying weaker cancellous bone. This speaks to the crucial need of developing techniques to combine improvements in cancellous bone healing or remodelling capacities together with augmentation of the mechanical support of implants such as bone screws in the surgical repair of hip fractures such as the femoral neck fracture.

1.2. Current Treatment Options

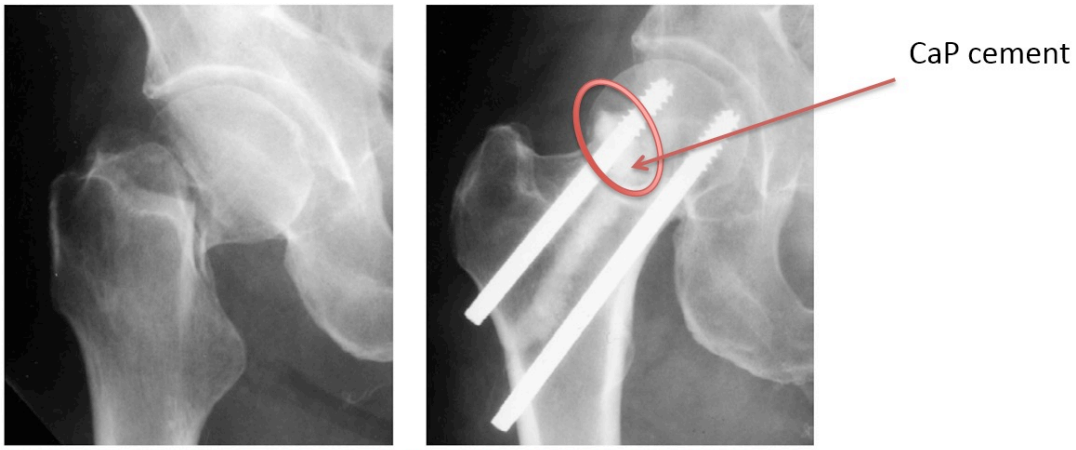
Many different treatments exist nowadays to control chronic Osteopenia and Osteoporosis (Cosman et al. 2014), although when fractures occurs in loadbearing bones a bio-mechanical solution is often preferred (Onnerfält 2010). With this goal in mind, surgeons usually use orthopaedic screws and plates in combination with other measures.

Metallic cancellous bone screws are prone to instability and failure when implanted in mechanically weak trabecular bone (Seebeck et al. 2005). This issue has several potential solutions, generally known as fixation augmentation or screw augmentation techniques (Moroni et al. 2006). One of the first such solutions applied was the injection of PMMA cements (Benum 1977), (Schatzker, Ha'eri, and Chapman 1978), (Struhl et al. 1990) and more recently resorbable Calcium Phosphate (CaP) cements (Kawagoe et al. 2000), (Procter et al. 2015), (Brown and Chow 1983), (Chow 2009), which have been shown to be more biocompatible and subsequently more adequate (Ooms et al. 2003). Furthermore, (Stadelmann et al. 2010) it has been demonstrated that CaP can compensate to some degree for the lack of bony support where the cortical bone is thin or weak.

1.3. Limitations of Current Treatment Options and Unmet Needs

Evidently each class of fracture may need a different treatment; hence a treatment that is successful for some particular cases might not be the most suitable for some others. This rational motivates scientists and designers alike to analyse the effectiveness and outcomes of every proposed treatment for each particular issue. Particularly, femoral neck fractures are the first major fracture to be treated with a device and in the 100 years since this innovation both mortality and morbidity have greatly decreased (Johansson et al. 2000), (Onnerfält 2010), however failures of screw fixation and also avascular necrosis remain unresolved despite the development of a wide range of implants and cement augmentation methods.

Many studies have demonstrated that CaP cements are beneficial for bone remodelling when compared with PMMA materials that are polymers that do not degrade (Kawagoe et al. 2000), (Ooms et al. 2003). However biodegradation and bone remodelling of CaP cements "*proceed at a slow rate*" (Ooms et al. 2003). Furthermore, (Mattsson and Larsson 2006) demonstrated that the use of CaP cements cannot be recommended for the particular case of femoral neck fracture, because they might affect negatively to the necessary revascularization of the damaged bone, increasing the risk of femoral head necrosis.



Displaced femoral neck fracture before (left) and after (right) internal fixation with cannulated screws and calcium phosphate for augmentation.

Figure 1. Femoral neck fracture fixed with CaP augmented cancellous screws (Mattsson and Larsson 2006).

1.4. Hypothesized Alternative

Recent studies (Kettenberger et al. 2015), (Hulsart Billström 2014) produced surprising results in control groups that demonstrated more rapid bone formation than expected. In both studies the overall objective was to evaluate delivery of materials that would favour osteogenesis. In both studies the control was a form of Hyaluronic acid gel in combination with HaP nanoparticles. In the words of Hulsart Billström (2014) "*Results and Conclusion: Nano-sized HAP formed bone with greater density compared with other HAP compounds. This could be due to the nano-sized HAP acting as building blocks for the mineralization*". In words of Kettenberger et al. (2015), *"An unexpected original finding […] was the rapid in vivo mineralization of the hydrogel […] independently of the bisphosphonate"*, when talking about hyaluronic acid with HaP nanoparticles. In other words, this type of biomaterial with HaP nanoparticles, that is initially soft and gel like, appears to be a more favourable environment to enable cells to integrate the biomaterial into new bone structure than an injectable CaP cement that sets like a solid block (Ooms et al. 2003). Consequently, such a hydrogel appears to function as a osteoconductive scaffold (Jeong et al. 2016) with the extra advantage of being injectable (Hulsart Billström 2014). This is sufficient evidence to propose it as an alternative to CaP cements for filling voids in the trabecular bone, especially where there is a risk of decreased local vascularity.

The disadvantage of a soft biomaterial like a hydrogel is poor mechanical properties as shown by (Jeong et al. 2016), while (Bakoš, Soldán, and Hernández-Fuentes 1999) defend it as the basis of mechanical improvements for bone implants. Which self-evidently would be an enormous advantage in AVN risk fractures (such as fractured neck of femur) if it could be used as a solution to improve the stability of cancellous screws without limiting the revascularization of the affected zone.

1.4.1. Results from a preliminary study of a HaP augmented hydrogel

A study was undertaken at EPFL (Monteiro Teixeira 2015) to evaluate the mechanically detectable effects of an HaP loaded hydrogel on screw pull-out in both synthetic bone substitutes as well as in bovine bone. The result of this study showed that there was no detectable augmentation effect in *synthetic urethane foam*, otherwise known as *Sawbones* (brand name). However the same study showed a strong trend of an augmentation effect with bovine vertebrae. This latter result was obtained using a 2 % Agar-Agar based hydrogel with a 40 % loading of hydroxyapatite particles in a pull-out force measurement of the augmented versus not augmented cancellous bone.

1.4.2. Precedent results of HaP particles on the bony screw pull-out strength

Another in-vivo study (Tami et al. 2009) showed the long term advantages of HaP particles loading around micro bone screws. In this study the HaP particles were added without using any hydrogel or cement. It was observed that the BMD was improved over time and that was correlated with the increasing in the pull-out strength. However it remained to be proven the mechanical improvement in the primary stability, which is the goal of this project.

1.4.3. Raft Foundations and Snowshoes simile

In order to explain what can account for the apparently paradoxical result of a gel being detectable using a mechanical test such as screw pull-out it is necessary to look at some examples of where this already occurs. For instance, in order to transfer the heavy structural loads of a building through to soft soil or even swampy ground with very low bearing capacity, civil engineers have developed the "raft foundation". In this the "Raft" can, "*spread the load imposed by a number of columns or walls over a large area, and can be considered to 'float' on the ground as a raft floats on water*" (Designing Buildings Limited 2016).

A similar principle is used in snow shoes, which spreads the body weight of a person over a much larger area than the shoe, allowing them to walk over a soft material like freshly fallen snow without sinking, a quality called "flotation".

Monteiro Teixeira's (2015) results suggest that greater loads can be transferred through a metallic screw in cancellous bone when the interface is augmented with a gel augmented with HaP. So it is very possible that a similar mechanism to that seen in raft foundations and snow shoes is operating where load transfers between a hard material (the screw) and soft material (the bone) are modified by a load spreading strategy. Following this rational, the hypothesis of the present study was formulated: *could it be proven, with statistically relevant level of confidence, that a soft biomaterial like a hydrogel with calcium particles (*HaP*) provide a mechanical improvement to the primary stability of cancellous bone screws?*

2. Materials and Methods

2.1. Main Study Design

Two sources of cancellous bone samples were used for this investigation; firstly bovine distal femurs and secondly human femoral condyles that were the test samples in two sub-studies. In both studies the bone samples used were in the form of cylindrical cores of 20 mm of diameter and 20 to 25 mm of length. A titanium cancellous bone screw (ref: 604026, Cancellous Screw, Stryker, Selzach, Switzerland) was inserted into a predrilled hole that was coincident with the axis of each cylindrical bone sample. In both sub-studies, the mechanical influence of different biomaterials on cancellous bone screw was tested. The method used to compare the mechanical properties of those biomaterials was to inject each biomaterial into the screw hole prior to the screw insertion and subsequent pull-out testing. The data from the pull-out testing and a µCT analysis of the trabeculae bone density were used to interpret the effect of the tested biomaterials. The general protocol is summarized in the flow diagram shown in Figure 2 and detailed in the following section.

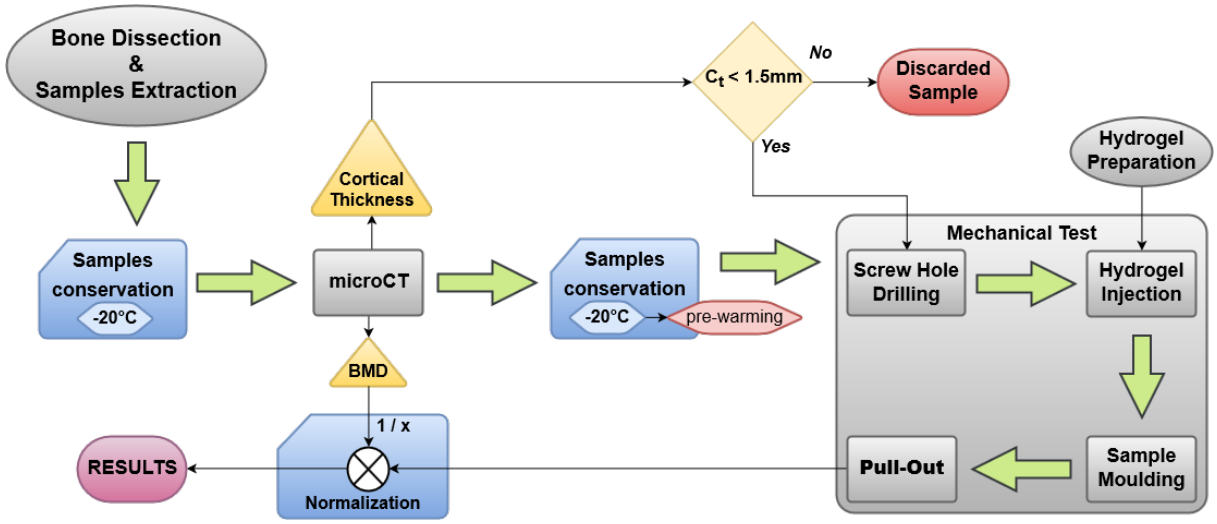


Figure 2. General process diagram. The "Mechanical Test" involves some steps that were done without interruption, without possibility of storing the samples in the freezer. The idea was to keep the sample as closer as possible to in-vivo conditions after the screw bone hole was drilled.

2.1.1. Bovine Study

The bovine bones were bought in a local slaughterhouse (Bell Suisse SA, Cheseaux-sur-Lausanne, Switzerland) the same day of the animal sacrifice. The bovine study included 5 experimental groups in which the only difference was the biomaterial filling the screw hole. In the first group, named Agar, the biomaterial was an hydrogel based on Agarose (ref: 05039, AgarAgar, Sigma-Aldrich, Buchs, Switzerland) mixed at 2 % (wt/vol); in the second group, named AgarHaP the same Agarose based hydrogel was used as carrier system to introduce a 40 % (wt/vol) concentration of hydroxyapatite particles – HaP here onward – (ref: 04238, Hydroxyapatite, Sigma-Aldrich, Leinfelden-Echterdingen, Germany); the third group, named Hyalectine, was injected with another hydrogel based on Hyaluronic Acid – HA here onward – (Hyalectine, Fidea, Italy) mixed at 3.5 % (wt/vol); the fourth group, named HyalectineHaP (abbreviated HyHaP in the figures) consisted in the same HA hydrogel used but charged with 40 % /wt/vol) of HaP; and the fifth group was named Control and any biomaterial was injected. Before drilling each screw hole, μCT scans of the cylindrical bone samples were performed to obtain trabecular BMD measurements for normalization of the results of the pull-out tests executed after

screw insertion. As the whole process always took longer than one day, the samples were stored at -20 °C until needed and later pre-warmed to about 37 °C before continuing as follows.

Group	Control	Agar	AgarHA	HyHaP
Biomaterial	void	Agarose	Agarose + HA	Hyaluronic Acid + HA

Table 1. Bovine group summary.

2.1.2. Human Study

The human cadaver specimens used in this investigation were obtained from either left or right femoral condyles from 6 different individuals from USA with no osteoporosis disease. All died between June and July of 2014 since when the femurs had been stored for over two years in a cryogenic freezer at -80 °C. At the moment of demise the individuals had an average age of 67.83 (range 32 to 84); an averaged weigh of 89.83 kg (range 68 to 143 kg) and with an averaged height of 172.67 cm (range 157 to 183 cm). The group consisted of four males and two females, Caucasian except for a single black male.

This sub-study included only 2 experimental groups. One had the screw hole filled with the Hyaluronic Acid hydrogel loaded with 40% of HA (Hy-HaP-H), as was done for the equivalent group HyHaP in the Bovine Study (section 2.1.1). The second group was filled with no material prior to screw insertion (control-H). Before the screw hole drilling, μCT scans of the cylindrical bone samples were performed for further analysis. The general protocol was similar to the one introduced in the section 2.1.1 with minor adaptations.

Group	Control-H	HyHaP-H
Biomaterial	void	Hyaluronic Acid + HA

Table 2. Human group summary.

2.1.3. Bovine Bone Samples Preparation

120 bovine samples were extracted from 13 adult bovine distal femur (condyles and trochlea). The samples extracted were cylinders with a diameter of 20 mm and not less than 20 mm length. First the bovine proximal femurs were dissected and kept at -20 °C in the freezer. Then, the bone cylinders were extracted using a crown drill mounted in a column drill machine (Bosch PBD 40, Leinfelden-Echterdingen, Germany), turning at 200 rpm and a band saw (Rexon BS-12RA, Taichung, Taiwan) at its slowest speed. The cortical layer was retained whilst the surface of the cortical bone was cleaned to remove soft tissue. This reduced the variance in the drilling and screw insertion depth.

Immediately after extraction, the samples were put individually in hermetically sealed standard laboratory Falcon tubes. The Falcon tubes were labelled with the original bovine femur identity, the extraction position in the distal femur, the sample dimensions and the date of extraction. The Falcon tubes were subsequently kept at -20 °C in the freezer.

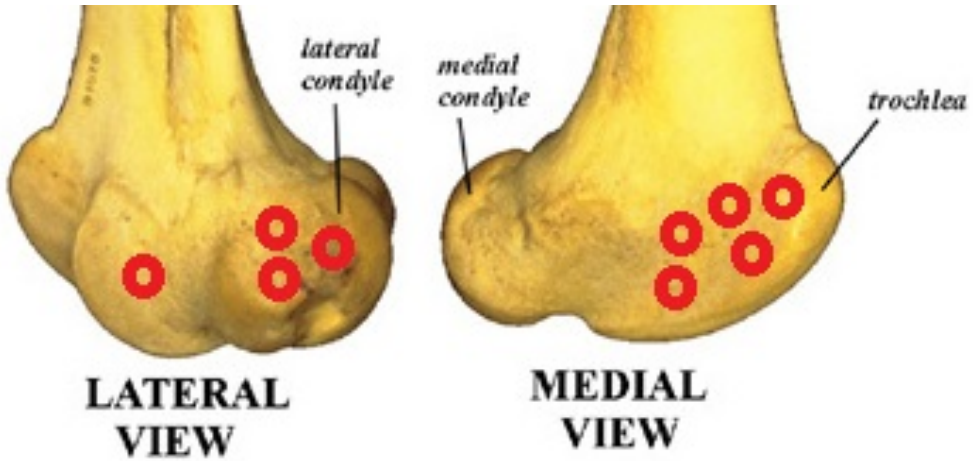


Figure 3. Bovine bone sample extraction sites.

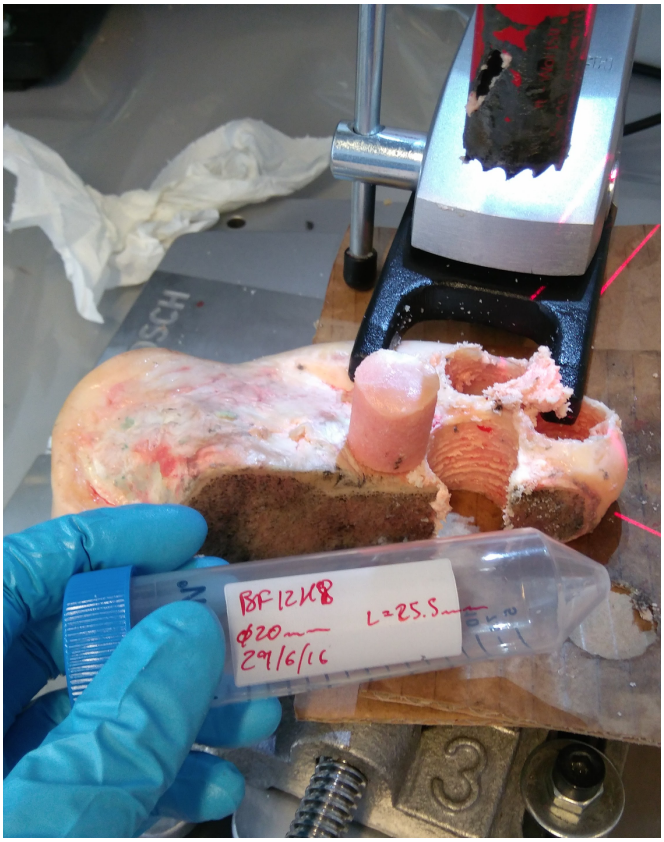


Figure 4. Example of bovine bone sample extraction.

2.1.4. Human Bone Samples Preparation

A total of 25 cylinders with the same dimensions as those used in the bovine study (section 2.1.3) were extracted from 6 human femoral condyles that were previously stored in a cryogenic freezer at -80 °C for over 2 years. Subsequently sample extraction was performed using a column mounted drilling machine (Bosch PBD 40, Leinfelden-Echterdingen, Germany) with a crown drill of 20 mm of internal diameter, turning at 200 rpm. In contrast to the bovine study, the human trabecular bone was simple to cut, allowing the author to perform the extractions with a manual metal saw instead of the

motorized band saw used to cut the bovine bones. After the extraction of the cylinders, each sample was tightly wrapped with standard laboratory plastic film (P7668-1EA Parafilm M, Sigma-Aldrich, Steinheim, Germany) to reduce the bone marrow substance loss and then put in standard laboratory Falcon tubes (hermetically closed) and kept in the freezer at -20 °C. The Falcon tubes were labelled with coded information about the original human femur identity, the extraction position in the distal femur, the sample dimensions and the date of extraction.

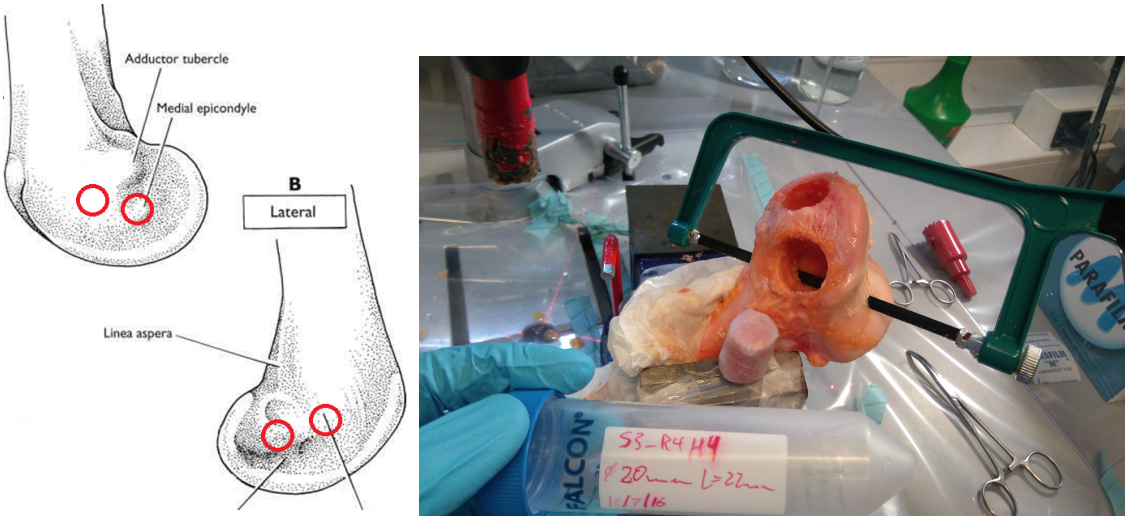


Figure 5. (left) Human scheme of the distal femur marking the sample extraction spots. (rigth) Human bone sample extraction example.

2.2. Hydrogels Preparation

Two kind of soft biomaterials loaded with HaP particles (Hydroxyapatite, Sigma-Aldrich, Leinfelden-Echterdingen, Germany) were evaluated. Firstly the concept was tested with a commercially available type of Agarose (ref: 05039, AgarAgar, Sigma-Aldrich, Buchs, Switzerland) $(C_{12}H_{18}O_9)_n$ – mixed at 2 % (20 mg per ml). Later, another commercially available hydrogel-like material based in hyaluronic acid (Hyalectine, Fidea, Italy) ($(C_{14}H_{21}NO_{11})_n$ with MW=800 KD), mixed at 3.5% (35 mg per ml) was used to confirm the results with an extended samples set. In both cases 40 % (400 mg per ml) of HaP particles were added to the hydrogels. The control groups contained 0 % of HaP, including one with only Agarose based hydrogel for the bovine study (see Agar-group in section 2.1.1).

Equation 1. Concentration in weight per volume.

$$wpv\ \% = \frac{solute\ (g)}{solution\ (ml)} x100$$

The Table 3 shows the different biomaterial composition used in each group of samples.

Table 3. Augmentation biomaterial components concentration per group.

Group	**Agar**	**Hyalectine**	**HaP**
Agar	2 %	-	-
Agar-HaP	2 %	-	40 %
Hy-HaP	-	3.5 %	40 %
Control	-	-	-

More information about the mixing protocol for each kind of hydrogel is in the Appendix C.2.

2.2.1. Characterization of the Hydrogel with Hydroxyapatite

"Rheology is an appropriate method for characterizing hydrogel mechanical properties" (Zuidema et al. 2014). Rheology tests were performed with the available rheometer (Digital Rheometer C-VOR, Bohlin Instruments Ltd – now Malvern Instruments Ltd, Dresden, Germany) in order to characterise the studied biomaterials sufficiently to enable further investigations or revisions of the current work. Following the protocol stablished by (Zuidema et al. 2014), both *Strain Sweeps* and *Frequency Sweeps* were performed to determinate the Strain and Frequency parameters needs to set the *Time Sweeps* that allows to find the hydrogel equilibrium modulus (Strain and Viscosity modulus).

A microscopy (Axiovert 100, Zeiss, Germany) was used to determine the size of the HaP particles used in this study. After dissolving a few milligrams of HaP (Hydroxyapatite, Sigma-Aldrich, Leinfelden-Echterdingen, Germany) in water a sample of the solution (< 1 ml) was put in a lab slide for microscopy and some pictures were taken for further analysis.

2.3. µCT Imaging and Processing

Micro-computerized tomography scans (μCT) (Skyscan 1076, Bruker µCT, Kontich, Belgium) were performed before drilling the screw holes to examine the condition of every bone sample (bovine and human). Three main aspects were analysed: the cancellous **bone mineral density (BMD)** that it is generally used as part of the diagnosis protocol for Osteopenia and Osteoporosis (Cosman et al. 2014) (see section 0), furthermore it has been shown that pull-out strength is linearly correlated to the BMD value (see section 3). Consequently, BMD was used as a normalization factor.

Every sample with **cortical shell thicker than 1.5 mm was rejected**. Based on a study (Seebeck et al. 2005) that demonstrated that the pull-out strength is dominated by the cortical shell when this is thicker than 1.5 mm. Finally, a **visual analysis of the reconstructed samples** was used to exclude samples with special features inside the trabecular structure such the grown line or irregular spaces, as samples with cortical surface so irregular or tilted that would compromise a proper pull-out test.

The samples were scanned in couples to reduce the scanning time. Therefore, two samples were placed into a unique Falcon tube for each μCT. To prevent bone samples from drying and loosening bone marrow substance, they were kept wrapped with standard laboratory plastic film (P7668-1EA Parafilm M, Sigma-Aldrich, Steinheim, Germany) during the full scan process and immediately brought back to the freezer at -20 $^{\circ}$C. Each scan single session (2 samples) extended for about 12 minutes.

2.3.1. Data Acquisition and Reconstruction

To achieve the analyses mentioned above the scan μCT was set with the following parameters: 1 mm aluminium filter, voltage 100 kV, current 100 μA, exposure time 120 ms, rotation step 0.5-degree angle, angle excursion 180-degree angle and spatial resolution of 35 μm. A calibration of the attenuation constant vs BMD was done for every scan session by scanning two cylindrical polymer-hydroxyapatite phantoms with a diameter of 16 mm and known mineral density of 0.25 g/cm^3 and 0.75 g/cm^3 in the same conditions as the bone samples. The projection images were subsequently reconstructed with NRecon and GPURecon Server (Bruker µCT, Kontich, Belgium). The beam hardening correction was automatically applied.

The trabecular BMD values were obtained from a cylindrical region of about 8 mm of diameter and 15 mm of length at the centre of every bone sample and below the cortical shell. That cylindrical region was defined large enough with the purpose of involving the volume that would be later occupied by the cancellous screw (4 mm diameter).

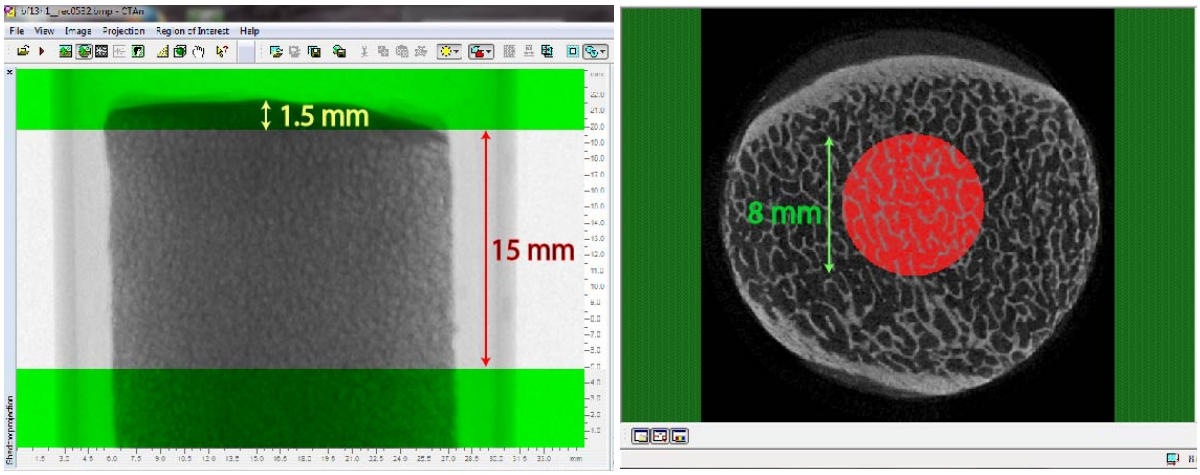


Figure 6. Reconstructed µCT scan of a validated bovine bone sample. The pictures show the region where the trabecular BMD was computed. This volumetric region is a cylinder that starts at 1.5 mm below the cortical surface and extends until 15 mm deeper with a diameter of about 8 mm.

At this moment the samples were analysed and validated or rejected and sorted in different testing groups as mentioned in section 2.1.1.

2.3.2. Hydrogel Distribution Analysis

Additionally it was necessary to understand the behaviour of the hydrogel once it was injected in a bone sample. More scans were performed in some of the validated samples to determine the final biomaterial distribution and to anticipate whether the hydrogel will be pushed through the trabecular bone structure or compressed between screw and screw hole walls. As this procedure was particularly time consuming and exposed the bone samples to an extra time at room temperature and hence more degradation, it was decided to limit the experience to a few bone samples. In consequence, those samples were later excluded from the main study to keep all the studied samples under the same testing conditions. With this goal, a first scan was performed immediately after the hydrogel injection and a second scan followed the screw insertion. Both scans where performed with the same parameters that were previously used for the BMD acquisition with the only exception of the angle excursion that was extended to 360-degree angle in the case of the second scan, to reduce the artefacts introduced by the metallic screw.

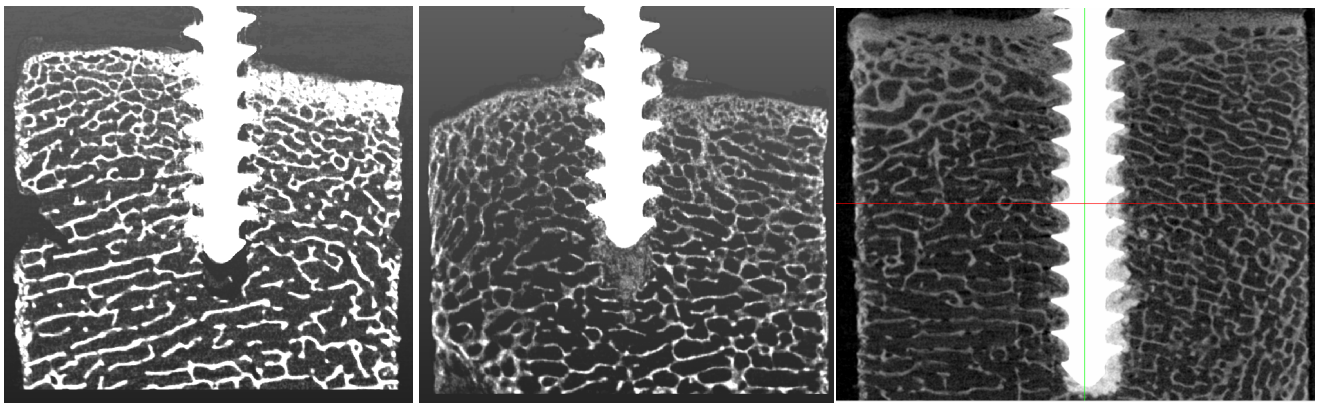


Figure 7. Samples scanned after screw insertion. From left to right: A) sample without any biomaterial injected. The screw hole limits are clear. B) Sample with hydrogel loaded with 40 % of HaP. The biomaterial is shown with the same grey values as the trabeculae. The interface between cancellous bone and hydrogel is not clear around the screw. C) Sample with hydrogel charged with 40 % of HaP and Iodine as contrast agent substituting 50 % of the bi-distilled H_2O. The hydrogel is clearly separable from the cancellous bone (see also Figure 14).

In some of those scans, the hydrogel used was prepared with a solution where 50 % of the bi-distilled water was replaced by a commercially available solution of Iodine (HEXABRIX-320, Guerbet, Roissy CDG Cedex, France). The iodine is a known contrast agent in X-ray tomography. More information in Appendix 0.

Supplementary single projections were taken for 20 minutes, after both scans, to resolve if the Iodine solution segregated from the hydrogel through the trabecular structure.

2.4. Cancellous Bone Screws Insertion

Apart from changing the material injected (see sections 2.1.3 and 2.1.4) the following procedure was unchanged for every sample from both studies and all the groups. Firstly, the samples were unfrozen/pre-warmed inside their Falcon tubes by plunging the tubes in water at a temperature between 37°C and 40°C. Then using the column drill machine mentioned above (Section 2.1.3), the specimens were drilled with a drill bit of 2.5 mm of diameter to a depth of 18 mm from the cortical surface always trying to keep a 90-degree angle between the drilling axis and the cortical surface. The screw hole depth was measured while drilling with the help of the available depth digital sensor embedded within the drilling machine. At this point the relevant biomaterial was injected through a special syringe (DISTRIP 1250, GILSON, France) within an inner-diameter smaller than 1 mm, for each group of samples. Between 0.1 ml and 0.3 ml of biomaterial was used to fill each screw hole until overflow was observed. Immediately after the screw was inserted. It was considered that the 18 mm depth had been achieved when the outside part of the screw was measured with a calliper to be 8 ± 0.1 mm from the cortical surface to the screw head top. Commercially available cancellous bone screws with 26 mm length, 4 mm outer diameter, 2.5 mm inner diameter were used (ref:604026, Cancellous Screw, Stryker, Selzach, Switzerland).

As the material under investigation in this project (the hydrogel-like soft biomaterial) does not set in a solid composite and the tests were performed carefully avoiding any mechanical damage for the screws, it was possible to clean them (with acetone and ethanol 70 %) after each test and then reuse them reducing significantly the number of screws needed and consequently the cost of the project.

2.5. Mechanical Test – Pull-Out

It was considered that the main question of this investigation could be resolved by the results of a mechanical test consisting in the application of an increasing tensile stress to the cancellous screw in the axial direction and measuring the maximum tensile strength applied at the precise moment when the screw is pulled out from the bone sample due to cancellous and cortical bone fracture, simulating implant loosening. This kind of test is well known as Pull-Out Test and is frequently used in biomechanical orthopaedics testing (see (Thomas, Pynsent, and Mccombe 1992) and more recently (Peter et al. 2004)). The standard that most closely matches this type of test is ASTM standard F543-07e1 (American Society for Testing and and American Society for Testing and Materials 2009) which describes and specifies the pull-out methods appropriate for testing Metallic Medical Bone Screws. Since this standard uses *synthetic urethane foam*, otherwise known as *Sawbones* (brand name), and our study was designed with cadaver bone sample, it was necessary to adapt the standard whilst following the overall philosophy of the cited document.

Before performing the pull-out test the samples were fixed with a fast curing resin (Technovit 3040, Wehrheim, Germany) as proposed by (Rincón-Kohli et al. 2009), into a specially designed mould that would be also used as holder during the pull-out test.

The mechanical tensile strength was applied by a tensile actuator available in the laboratory (ref: E3000KZ832, Instron 3000, USA) at a rate of 5 mm/min, between the bone sample holder and the screw head holder. And the data were acquired with an axial strength transducer (ref: 66901, Dynacell Dynamic Load Cell 5 kN, Instron, USA) and automatically saved in an excel file. Later the data were processed with Matlab to obtain a single pull-out force value per sample tested (in Newtons) and the energy to failure (measured in Jules). The pull-out force was measured as the maximum tensile strength at the moment of bone failure. The energy to failure is the area covered by the function of tensile strength before the bone failure (see Figure 10. Example of typical pull-out test data. During the pull-out test the tensile load applied at the screw head increases approximately linearly until failure occurs as the screw strips bone. Then the displacement continues, and the load drops due to the lack of resistance. The area under the load curve until the bone failure is the *energy to failure* and the pull-our strength is the maximum load value, which is achieved at the moment of bone failure.).

3. Results

3.1. Rheology of the biomaterials studied

The characterization of the biomaterials loaded with HaP was performed with the rheometer available in the laboratory (Digital Rheometer C-VOR, Bohlin Instruments Ltd – now Malvern Instruments Ltd, Dresden, Germany). The initial parameters of Strain = 0.02 and frequency = 5 Hz were obtained by previous Shear Sweep and Frequency Sweep test. With these values a Time Sweep was performed acquiring the values resumed in Table 4. It can be appreciated that both biomaterials had a similar viscosity.

Table 4. Rheology acquired with Time Sweep at frequency of 5 Hz and Strain of 2 %.

Hydrogel	Viscosity Modulus (Pa)		Shear Stress (Pa)		Elastic Modulus (Pa)	
	Mean	STD	Mean	STD	Mean	STD
HyHaP	4300.67	1024.35	310.41	19.99	5259.87	1396.38
AgarHaP	4179.17	849.04	118.42	83.74	20591.89	3818.74

3.2. Microscopy to size the HaP particles

The pictures taken with the camera installed on the lab microscope (Axiovert 100, Zeiss, Germany) showed an acceptably homogeneous particle size of about 3 micrometres.

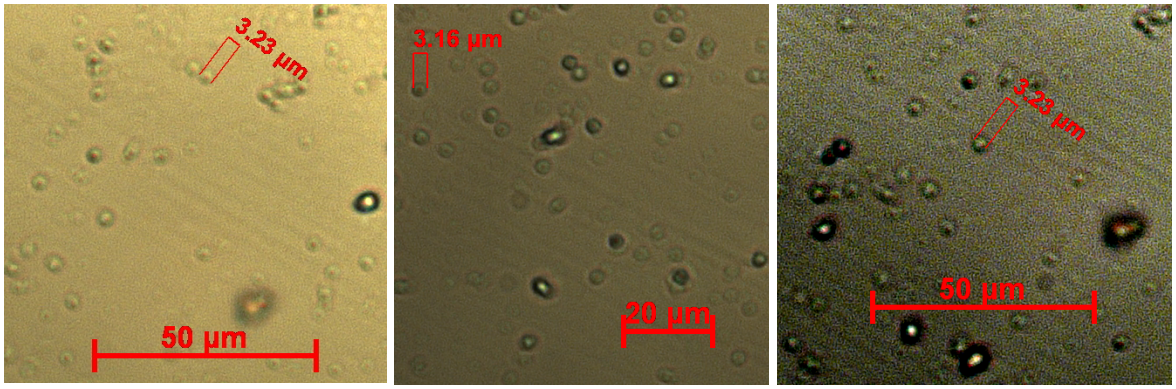


Figure 8. Pictures from microscope Axiovert100, lens x32. HaP particles are visible and measured about 3 µm.

3.3. Bovine Study

3.3.1. µCT Acquisition of BMD Values

The BMD derived from the μCT scans confirmed that the lowest BMD values of the bovine bone samples –total range was 0.0908 to 0.3538 g/cm^3– were within the range that a recent study (Maeda et al. 2011) found in people with femoral neck fractures, as shown in Table 5.

Table 5. BMD values per group of bovine study vs human data from Maeda et al. (2011). (BMD in g/cm^3)

Group	# samples per group	BMD			From Table 2. (Maeda et al. 2011)	
		Mean	STD	Range		
Control	15	0.21592	0.07848	0.09 to 0.35	BMD at femoral head	0.1273 ± 0.0294
HyHaP	12	0.20759	0.08148	0.11 to 0.33		
AgarHaP	13	0.20089	0.09216	0.09 to 0.35	BMD at femoral neck	0.0888 ± 0.0451
Agar	11	0.19589	0.06569	0.104 to 0.3		
Hy	12	0.18643	0.07336	0.08 to 0.3		
Total	**38**	**0.20212**	**0.07709**	**0.09 to 0.35**		***Mean ± STD***

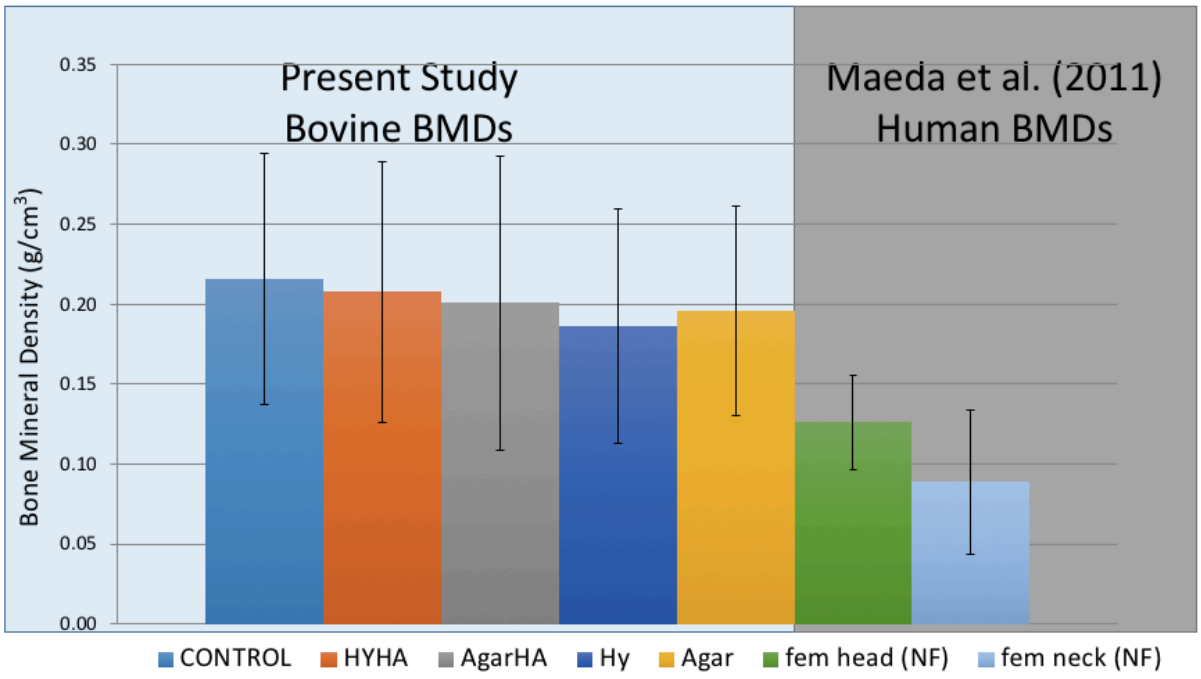


Figure 9. BMD values per group of bovine study vs human data from Maeda et al. (2011). (BMD in g/cm^3)

3.3.2. Maximum Pull-Out Forces

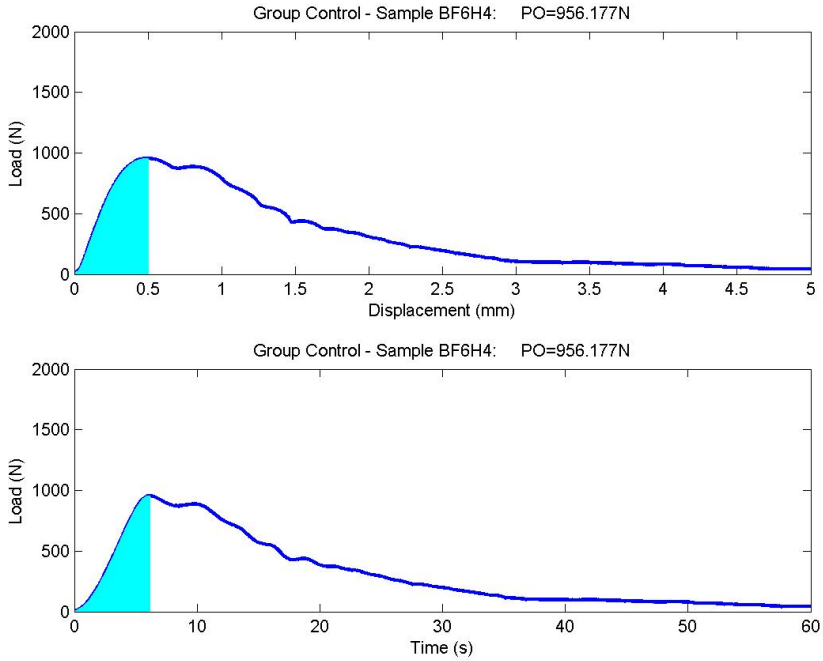


Figure 10. Example of typical pull-out test data. During the pull-out test the tensile load applied at the screw head increases approximately linearly until failure occurs as the screw strips bone. Then the displacement continues, and the load drops due to the lack of resistance. The area under the load curve until the bone failure is the *energy to failure* and the pull-our strength is the maximum load value, which is achieved at the moment of bone failure.

Main result

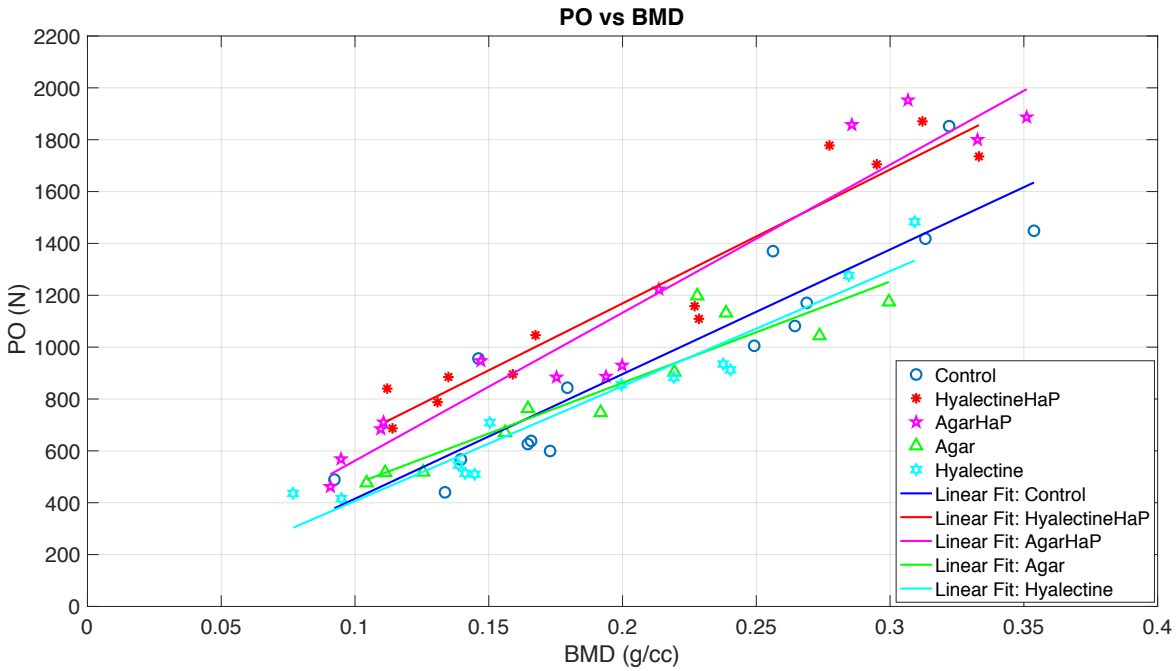


Figure 11. Pull-Out maximum forces measured in Newtons vs the BMD values measured in g/cm3 by µCT. Each marker type denotes a group of samples and each coloured line corresponds to the linear fitting of group of samples with the same colour, as indicated in the figure legend.

The pull-out forces obtained have been arranged in groups, as explained in section 2.1.1, and linear fitting curves have been computed (see Figure 11). When comparing the Control group (no material injected) with the HyHaP group (hyaluronic acid with HaP) the linear fitting functions that correspond to the data sets have approximately similar slopes:

$$\mathrm{PO}_{Control}(N) = 4805.4\ x\ BMD\left(\frac{g}{cm^3}\right) - 65.113 \rightarrow Slope_{Control} = 4805.4\ (Ncm^3/g)$$

$$\mathrm{PO}_{HyHaP}(N) = 5161\ x\ BMD\left(\frac{g}{cm^3}\right) + 136.59 \ \rightarrow \ Slope_{HyHaP} = 5215.8\ (Ncm^3/g)$$

Where PO is the fitted pull-out force for each group. However, the linear curves are not exactly parallel, the HyHaP group fitting is only inclined 8.54% more than the Control group. As a result, for the range of BMD studied, the samples injected with HyHaP showed a net augmentation effect of more than 200 N. Moreover, this apparently constant increase in the pull-out strength means that an inverse relation exits between the BMD of the sample and the augmentation effect achieved with HyHaP. This was confirmed by computing the relative difference between the mentioned linear fitting functions, which showed a negative exponential relation between BMD and HyHaP augmentation effect (see Figure 12).

$$Augmentation\ function\ from\ Control\ to\ HyHaP:$$

$$f(BMD) = 100\frac{PO_{HyHaP} - PO_{Control}}{PO_{Control}}\ (\%)$$

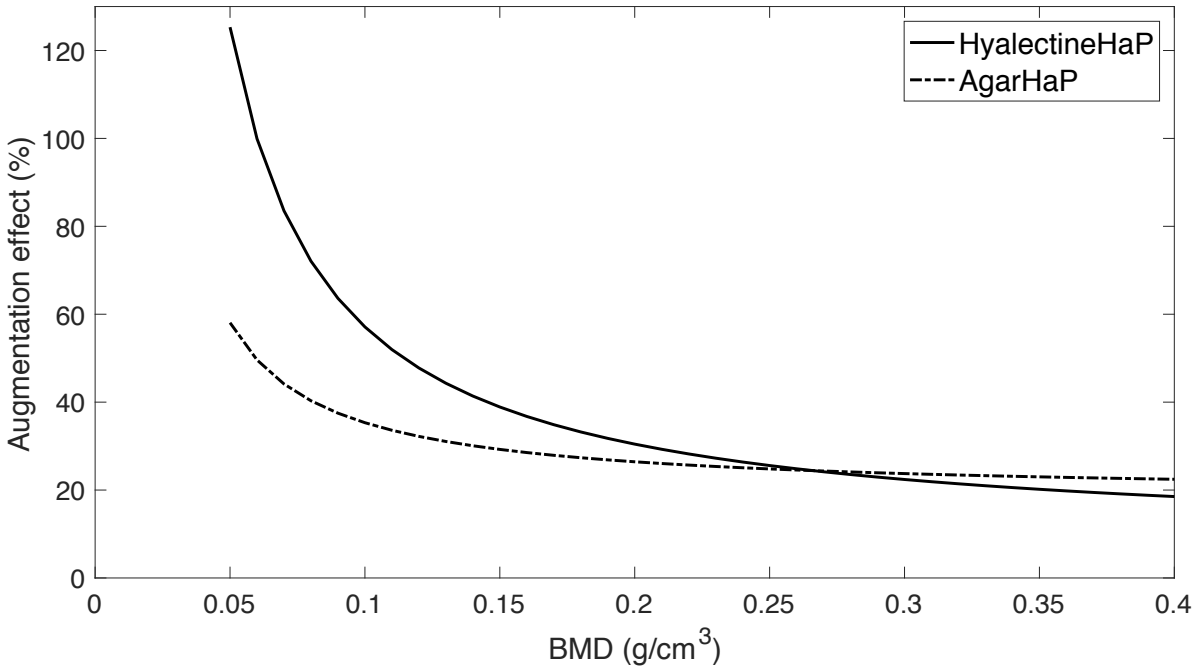


Figure 12. The relative difference between the augmented groups (those loaded with HaP) and the non-augmented group (the group Control as reference) is the increase in force achieved with the augmented groups when compared with the non-augmented (%). See the equation "augmentation function from Control to HyHaP".

The statistical comparison between the studied groups with biomaterials and the reference group Control, showed a significant difference in the normalized pull-out strength between Control group and both groups with HaP: HyHaP (p_{value}=0.00011) and AgarHaP (p_{value}=0.00063). As can be observed in Figure 13.

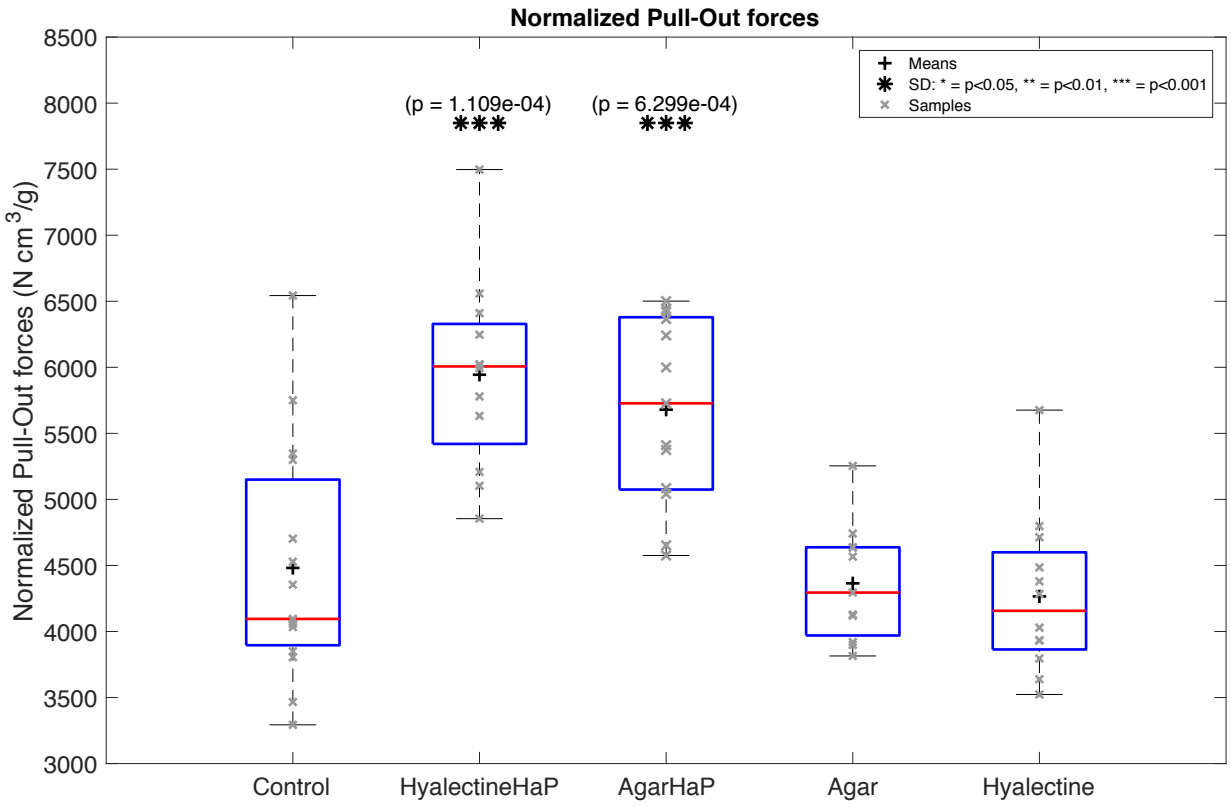


Figure 13. Statistical comparison of normalized pull-out strengths. Blue boxes represent 25th and 75th percentiles; Grey horizontal bars are minimum and maximum values; Red bars show the median; Grey markers "x" represent the normalized pull-out strength for each sample of each group; Black markers "+" represent the means.

3.4. Human Study

In the case of the human bone samples, the μCT scans revealed that the cadaveric femurs had suffered serious degradation during the low temperature storage. The reconstructed images (see Figure 14-left) showed the presence of voids in-between the trabeculae instead of the expected bone marrow substance. These unexpected voids allowed the hydrogel to disperse widely into the trabecular structure instead of remaining local to the screw (see Figure 14-center & right). This negative result was such a major departure from the anticipated test conditions that no further human testing was undertaken. Consequently, this part of the study was abandoned.

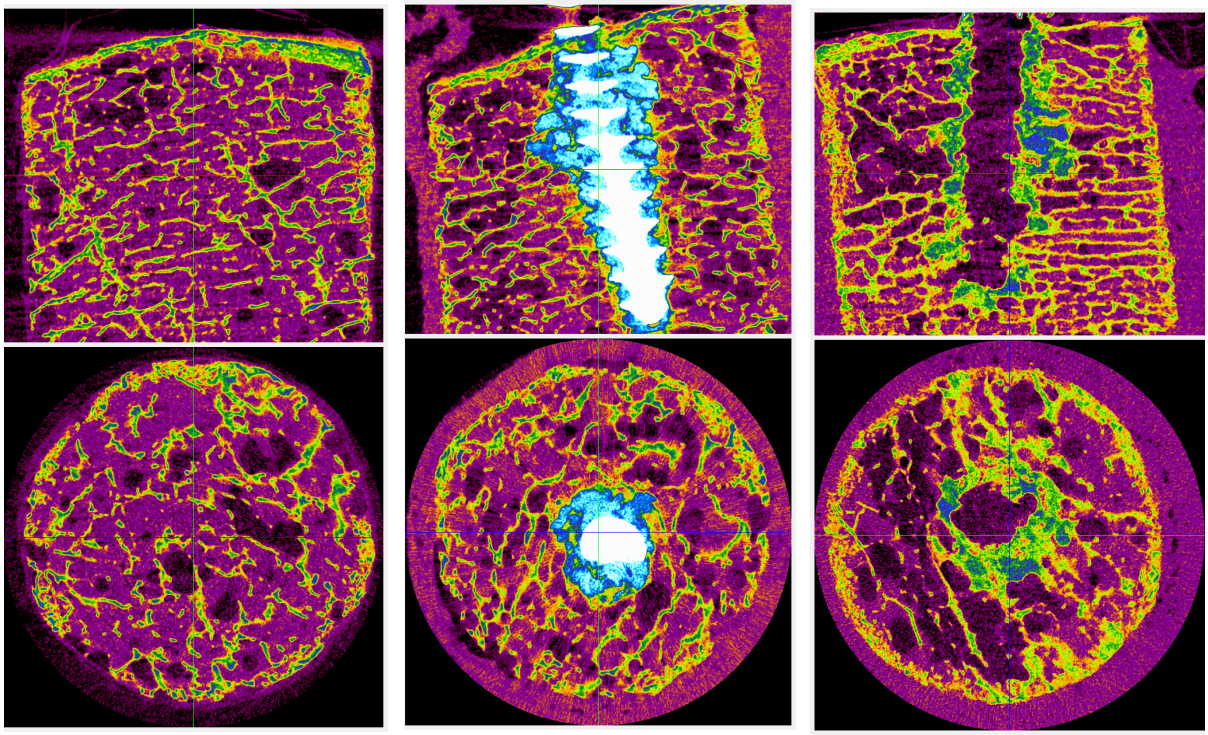


Figure 14. Reconstructed images of human bone samples. (Left) one of the first scans planned to obtain the trabecular BMD value revealed the presence of gas in-between the trabeculae. (Centre) sample with cancellous screw already inserted and previously injected with HyHaP and Iodine contrast agent. The hydrogel segregates through the trabeculae. (Right) after performing the pull-out test, a significant volume of hydrogel remains in this human sample, outside the screw diameter, penetrating deep into the trabeculae. (All) Colours note: pink shades show the soft tissue, including bone marrow substance and PBS; yellow and orange show the mineral inside the trabecular structure; blue shades are mostly due to the iodine dissolved in the hydrogel; the white is due to the metal present in the screw.

4. Discussion

Two pre-existing studies suggested that the combination of HaP nanoparticles suspended in hyaluronic acid produced favourable conditions for new bone formation. The material used in both studies was soft and gel like and did not have intrinsic mechanical stability. The main goal of the present study was to determine whether or not this form of non-setting biomaterial is able to provide primary mechanical stability of cancellous bone screws. The evidence of soft material mechanical properties in fields like civil engineering (Balkema 1996), (see section 1.4.2) concurrently with the possibilities as bone implant that Bakoš, Soldán, and Hernández-Fuentes (1999) predicted for hydrogels carrying hydroxyapatite contributed to motivate the current investigation. The method chosen to quantify mechanical stability was a screw pull-out test, a method frequently used to test bone screws and bone cements (Thomas, Pynsent, and Mccombe 1992), (Procter et al. 2015), (Kawagoe et al. 2000), (American Society for Testing and and American Society for Testing and Materials 2009).

The pull-out results normalized with the trabecular BMD values previously measured showed a significant difference ($p - value = 1.1 \cdot 10^{-4}$) between the Control group (non-augmented) and the HyHaP group (augmented hyaluronic acid at 3.5 % loaded with 40 % of HaP). This is shown in Figure 12. This result confirms that there is a mechanically detectable improvement in screw pull-out due to the presence hydroxyapatite particles in the hydrogel. In the same manner a significant difference ($p - value = 6.3 \cdot 10^{-4}$) was found between the Control group and the AgarHaP group (augmented with agarose at 2 % loaded with 40 % of HaP). However, the composition of both biomaterials was initially different, is evident that the presence of HaP particles produces the mechanical improvement.

Adult bovine distal femur was the main material used in this investigation. In a previous part of the present study, bovine proximal tibial samples were tested and discarded from the main study because their averaged BMD values were much higher than those usually expected in humans ($< 0.4\ g/cm^3$ for femoral cancellous bone). For the same reason, young bovine femurs samples were finally excluded as well for this current work. However, these previous extractions, the BMD measurements and the tests performed with the excluded samples were useful to validate the testing method and protocols (appendix A).

The results obtained with the samples accepted fit with a linear dependency between the measured BMD values and the pull-out forces (see Figure 11). This result confirms BMD as an important parameter related to pull-out forces and a convenient normalization factor. However, as Figure 11 shows there are some samples well outside the linear fitting line and it could be that this is due to the effect of cortical shell thickness differences. As introduced in section 2.3, the cortical shell thickness was used as a sample exclusion criterion. This is because Seebeck et al. (2005) demonstrated that cortical shells thicker than 1.5 mm are responsible of more than 50 % of the pull-out strength, hence any change of the cancellous bone mechanical properties could be obscured by the cortical shell. For that reason, the first criterion of exclusion was to avoid samples with cortical shell thicker than 1.5 mm. Although this limit should guarantee that the pull-out strength measured was dominated by the mechanical properties of the cancellous bone bellow the cortex, the contribution of the thin cortical shell must not be ignored. Actually, a detailed analysis of two of the samples used in this study confirmed this hypothesis. It was found that two samples with similar trabecular BMD values but very different cortical shell thickness (both under 1.5 mm) showed an important difference in their pull-out strength. As that was done only for a couple of samples it cannot be assumed as a proven theory, so it remains to be confirmed by further testing. It could be presumed that the trabecular bone contribution to the pull-out strength might be isolated by removing the cortical shell previously to the mechanical test. However, this procedure is, apparently, unlike to be followed in any real surgery, so we understood that the most general situation should include the effect of the cortical shell.

The present study has a number of limitations. First, the two soft materials used for this study, agar and non-crosslinked hyaluronic acid, only served as model substances and were chosen because of

their simple preparation and handling. Agar is a hydrogel with a 3-dimensional molecular network that is crushed into pieces when it is deformed, for example during injection. Non-crosslinked hyaluronic acid, in contrary, is a viscous fluid which is freely deformable due to a lacking polymer chain network. Non-crosslinked hyaluronic acid is characterized by a very high turnover in the human body and would be resorbed *in vivo* within several days (Stern 2004). For an *in vivo* application, it is debatable if a non-cross-linked, quickly resorbable material is sufficient as transport medium for the hydroxyapatite particles or if a longer persisting, cross-linked matrix would be needed. It is unclear, if and how a rapid disappearance of the matrix influences the implant stability and the biological response over time. The presented experiments can only capture the situation immediately after screw insertion and animal tests would be needed for an evaluation of the temporal development of the implant integration and stability.

Other limitations include the bad conservation of human femurs that were supposed to confirm the results with bovine samples. Further preclinical testing must avoid repeating this problem by acquiring fresh bone samples or improving the conservation system. The distribution of the biomaterial inside the screw hole was analysed with the help of the Iodine, however it was impossible to determinate the distribution of the hydroxyapatite alone, which could help to explain the mechanism that produce the pull-out strength increase. Histological analysis of the samples after the screw insertion might help to complete the knowledge about the distribution of the hydrogel and the HaP. Unfortunately, we did not find an established protocol to perform histology that could guarantee a relevant result with the particularities of our study. This protocol remains to be developed for further analysis of the augmentation mechanism. Another issue was related to the used HaP particles size which has been shown to be one order of magnitude larger than what is accepted as nanoparticle (1 to 100 nm) (AFNOR 2015).

The range of BMD studied (0.0908 to 0.3538 g/cm^3) was chosen by purpose to be low enough to be comparable with human cancellous BMD (i.e. Wachter et al. (2001) reported 0.128 to 0.31 g/cm^3 in human proximal femur), although it is low when compared with the 0.31 to 0.87 g/ cm^3 reported by Han et al. (1996) or the averaged 0.6 gm/cm^3 found in more recent literature (Liu, Niu, and Wang 2012). We realised that the way in which BMD measurements are reported is quite confusing, depending on the literature. Per instance, another study (Keaveny et al. 2001) reported a very large range in apparent density (0.1 to 0.7 g/cm^3), which is a volumetric percentage of bone but reported in the same units as BMD. There are many articles (Houde et al. 1995), (Nakavachara et al. 2014) that reported values of BMD per area (in g/cm^2) because it was measured with techniques like DEXA, instead of using volumetric scans like CT or CBCT. Actually, it is not a new issue in the literature as, in words of (Ott et al. 1997), "*Kazman et al. focused attention on the issue by describing a calculated volumetric bone mineral density they termed "bone mineral apparent density (BMAD)*" (KATZMAN et al. 1991).

Regarding the use of μCT imaging to analyse the hydrogel distribution around the screw: since the hydroxyapatite and the bone have a similar attenuation coefficient for the X-rays, it become necessary to add a contrast agent to the hydrogel in order to identify the interface between both. For that reason, the hydrogel used for these scans was prepared with a solution where 50% of the bi-distilled water was replaced by a commercially available solution of Iodine (HEXABRIX-320, Guerbet, Roissy CDG Cedex, France). Iodine also helped to confirm the existence of voids in the trabeculae of the human samples.

The results suggest that mechanical stabilisation of an orthopaedic implant device such as a screw can be obtained by increasing the HaP content to 40 % in the hyaluronic acid gels tested. Even though the viscous solution was highly loaded with particulate it remained injectable through a relatively small needle diameter (1.1 mm). It is feasible to make an injectable gel like biomaterial that can load bear. Using the linear curves as a reference (Figure 11), the absolute difference between test and control groups is typically at least 200 Newtons. This is comparable to the increase in pull-out force (approx.

100 Newtons at 5 days) demonstrated by Larsson et al. (2012), in CaP augmentation of 4 mm diameter screws in an in-vivo lapine model. The CaP cement in the latter study is approved for augmentation of cancellous screws in cancellous bone and sets shortly after injection into bone. However, sometimes this kind of cement sets prematurely making injection impossible and so an injectable gel as proposed in the present study could be advantageous as it remains injectable for longer working periods.

The studies of Kettenberger et al. (2010) and Hulsart Billström (2014) showed favourable new bone formation in a similar combination of Hyaluronic acid but at much lower HaP loadings and HaP particles at least 10 times smaller. Hence, it remains to be proven in further preclinical testing that the level of loading used in the present study would still show such favourable biological new bone formation response in cancellous bone but with a much higher quantity of HaP and a different particle size.

5. Conclusions

Two types of soft biomaterials, agar as hydrogel and hyaluronic acid as viscous fluid, were used pure or loaded with 40 wt/vol % of HaP to fill the pre-drilled holes before screw insertion. Unfilled holes served as control. The results showed that the pure materials did not have any effect on the primary stability of the screws, the HaP loaded materials, however, significantly increased the pull-out strength. This augmentation effect was independent from the type of soft material used and increased with decreasing BMD. Consequently, it was shown that a soft biomaterial, when loaded with sufficient HaP (40 %) remained injectable and at the same time provided initial mechanical stabilisation of an implant in bone as measured by a screw pull-out test. This is a result that has not been previously presented in the published biomaterials literature. Furthermore, this opens the possibility of combining the long and short-term advantages of using not setting biomaterials with HaP.

Finally, the originality of the present study is to combine HaP and a soft, non-setting biomaterial. The aqueous, soft matrix makes the material injectable through a small needle and would significantly facilitate the application in clinics. Depending on the type of biomaterial used, the matrix itself can have further benefits. Hyaluronic acid, for example, is known to support healing processes and to be bacteriostatic and anti-inflammatory (Carlson et al. 2004; Dahiya and Kamal 2013).

The combination between hydroxyapatite and hyaluronic acid has also been shown to induce a rapid in vivo mineralization leading to the in situ formation of an osteoconductive scaffold when applied into poor quality, peri-implant bone (Kettenberger et al. 2015). However, it has to be noted that this study was performed with a much lower HaP content than the present study. A similar positive biological effect with a HaP content of 40 wt/vol % in soft materials remains to be verified in an in vivo study.

6. Acknowledgments

It is a pleasure to thank Professor Dominique Pioletti for giving me the opportunity of developing my Master Project in his laboratory.

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Appendixes

A. Poster presented at the World Congress of Biomechanics, Dublin 8-12 July 2018

Non-setting hydrogels containing particulate hydroxyapatite can increase primary stability of bone screws in cancellous bone

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INTRODUCTION

Fracture fixation in weak bone is still a clinical challenge. Augmentation of screws was shown to increase their primary stability. The currently used calcium phosphate (CaP) or polymeric bone cements, however, present important drawbacks such as induced toxicity and/or impaired bone neo-formation. In particular the use of injectable CaP resulted in impaired blood supply in femoral neck fractures [1]. A **new approach** to enhance bone screw primary stability without affecting the bone formation is the use of non-setting calcium phosphate loaded soft materials as the augmentation material.

HYPHOTHESIS

Do soft biomaterial like hydrogel or viscous liquids containing ***hydroxyapatite*** particles (**HaP**) enhance the primary stability of **cancellous bone screws**?

OBJECTIVES & CONDITIONS

- (i) Controlled bone mineral density (**BMD**) and screw positioning
- (ii) Increased pull-out strength with non-setting injectable biomaterial
- (iii) Statistical significance
- (iv) Extrapolation to human BMD values

MATERIALS AND METHODS

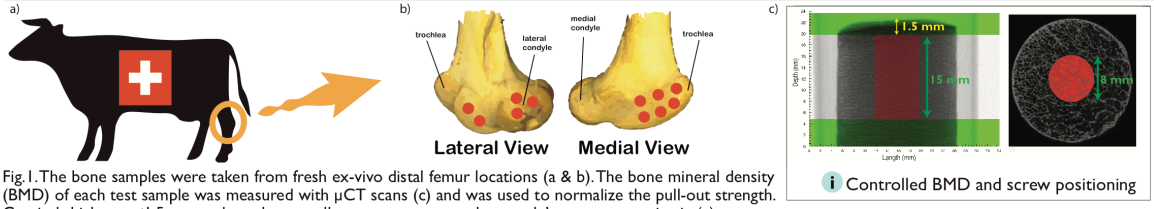


Fig.1. The bone samples were taken from fresh ex-vivo distal femur locations (a & b). The bone mineral density (BMD) of each test sample was measured with µCT scans (c) and was used to normalize the pull-out strength. Cortical thickness <1.5 mm and regular cancellous structure were the sample's acceptance criteria (c).

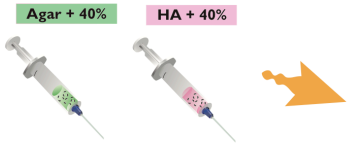


Fig.2. **Two** types of **biomaterials** (non-crosslinked hyaluronic acid (HA) as viscous fluid and agar as hydrogel) were **loaded with 40 wt/vol % of HaP** and characterized.

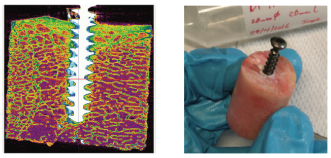


Fig 3. A second µCT scan (with iodinated contrast agent added to the injected material) allowed as to visualize the material after screw insertion.

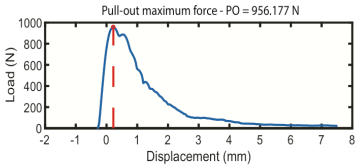
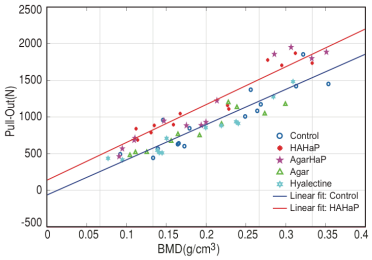


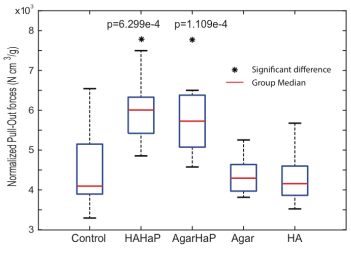
Fig 4. The screw augmentation effect of all materials was evaluated through pull-out tests in bovine cancellous bone and compared to the non-augmented situation (control).

RESULTS

ii Increased pull-out strength



iii Statistical significance



iv Extrapolation to human BMD values

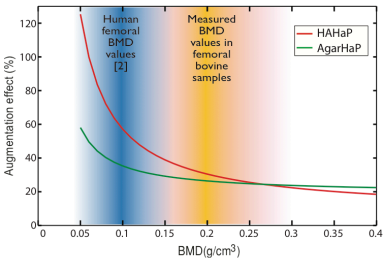


Fig.5 & 6. Both materials loaded with HaP increased the normalized pull-out strength of the screws compared to control samples and particle-free materials. This counter-intuitive augmentation effect increased with decreasing bone mineral density (Fig.7) and was independent from the type of the soft materials used.

Fig.7. The augmentation effect of the AgarHaP and the HAHaP groups (relative difference of normalized pull-out strength functions with Control group as reference) in relation to the BMD.

DISCUSSION

We were able to demonstrate that non-setting injectable biomaterials loaded with ceramic particles can significantly enhance the primary stability of cancellous bone screws. This material combination opens the unique possibility to achieve a screw augmentation effect without impairing or even potentially favoring the bone formation in proximity to the screw. This effect would be particularly advantageous for the treatment of osteoporotic bone fractures requiring a stabilization with bone screws.

CONCLUSIONS

- Soft materials with 40 wt/vol % HaP particles increase bone screw primary stability.
- Augmentation effect was not dependent on material type but on presence of HaP.
- Tested materials are injectable through a cannula with a diameter smaller than 1 mm.
- Injectable materials with HaP are proposed as new strategy for screw augmentation in weak bone.

ACKNOWLEDGEMENTS

Our acknowledgement for their interest as for supplying the hyaluronic acid to Fidia Pharmaceutici S.p.A.

References:
[1] Mattsson, P., Alberts, A., Dahlberg, G., Sohlman, M., Hyldahl, H.C., Larsson, S. 2005. "Resorbable cement for the augmentation of internally-fixed unstable trochanteric fractures A PROSPECTIVE, RANDOMISED MULTICENTRE STUDY. J Bone Joint Surg Br 8787, 1203–9.
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B. Protocols

B.I. Samples Extraction

Materials

- Band saw (Rexon BS-12RA, Taichung, Taiwan)
- Column drill machine (Bosch PBD 40, Leinfelden-Echterdingen, Germany)
- Crown drill (25 mm outer diameter, 22 mm inner diameter)
- Protection equipment (mask, security glasses, dissection lab coat, lab globes)
- Dissection kit: Scalpels (big size blades), clamps, scissors, etc.
- Cardboard (approximately 20x20 cm)
- Lab tray big enough to be a temporal recipient for the bovine bone before been cut
- Surfaces protection paper
- 1 L of saline solution (PBS at 10%)
- Distal Femoral Bovine specimen
- Digital camera
- Scale
- 50 ml lab tubes (Falcon: 25 mm inner diameter)
- 2 studio tables

Conditions

- The whole procedure was done in the P2 Lab of LBO EPFL, 1015 Lausanne, Switzerland
- Room temperature

Procedure

Specimen preparation

1. The bovine bone specimen should be as clean as possible of soft tisue.

Column Drill preparation

2. Set drilling speed at 200 rpm
3. Fix the crown drill
4. Switch on the light and the laser assistant for centring

Band Saw preparation

5. Protect the working plate with some protecting paper to avoid extra cleaning
6. Set the speed at minimum
7. Check that the saw mounted is adequate for bone or wood (big and few tooth)

Pre-cutting procedure

8. With the band saw, cut the bone transversely to remove the useless diaphasic bone.

9. Rely the transversal plane created with the precedent cut on the working plate of the band saw and cut the distal bone by the sagittal plane passing in between the distal condyles and continuing by the middle of the trochlea (see Figure 15 and Figure 16)
10. Save one side for later use in the plastic try with the litter of saline solution
11. Use the sagittal plane created as base for the following work

Cylinders extraction

12. Select appropriate area for drilling depending on the cortical surface tilt and regularity
13. If necessary use the band saw to cut a slice of bone in the opposite side of the desired drilling area, in order to create a base perpendicular to the desired drilling direction.
14. Fix the bone specimen with the clamp attached to the column of the drilling machine (see Figure 17)
15. Drill with to a minimum depth of 30 mm
16. Cut lower end of the cylinder with the band saw (Figure 18)
17. Use the scale or calliper to measure the length of the cylinder
18. If necessary cut the cylinder to reach a length in the range 22 to 25 mm
19. Discard the cylinder if the length is lower than 20 mm
20. Put the cylinder in a Falcon tube and store it in appropriate freezer at -20 °C
21. Repeat from step for the remaining extractions

Notes about preferable extraction zones

22. The cortical shell is thinner at the trochlea than at the condyles
23. The medial condyle has the most dense trabecular bone
24. Surfaces covered by cartilage have such a thin cortical shell that it can be removed with scalpel

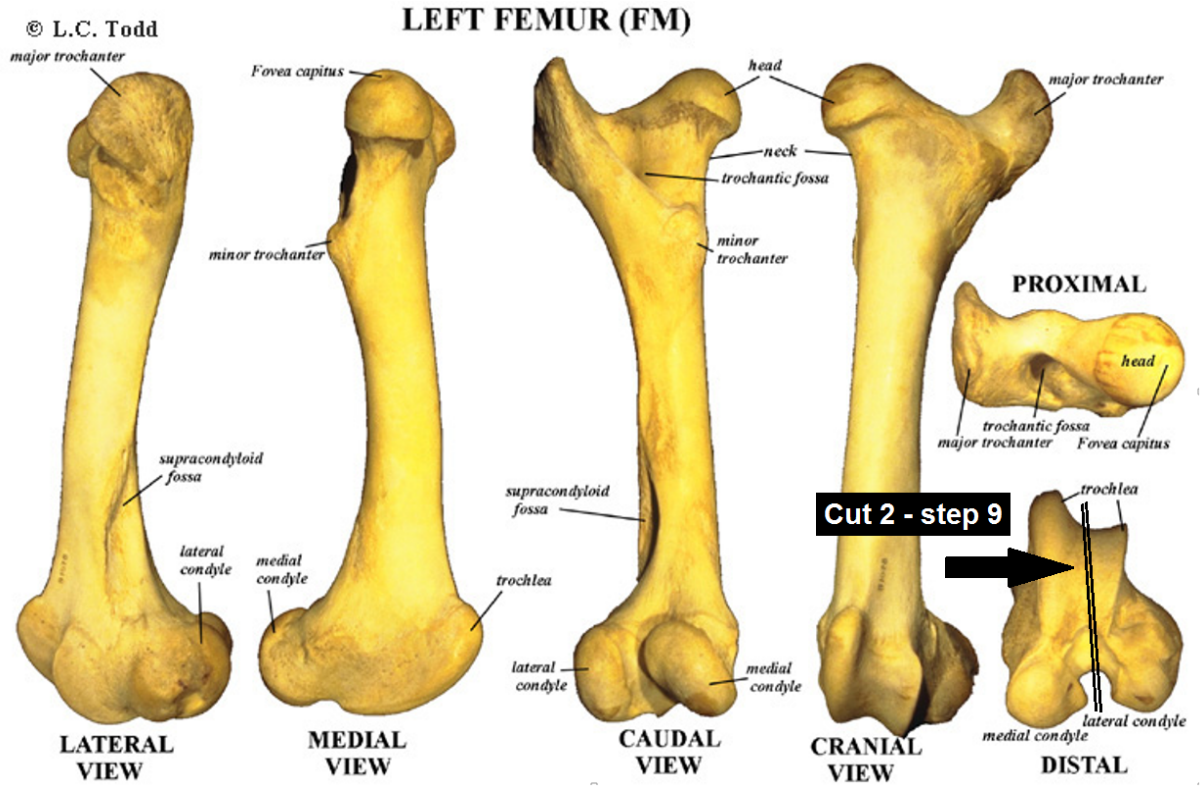


Figure 15. Bovine femur physiology.

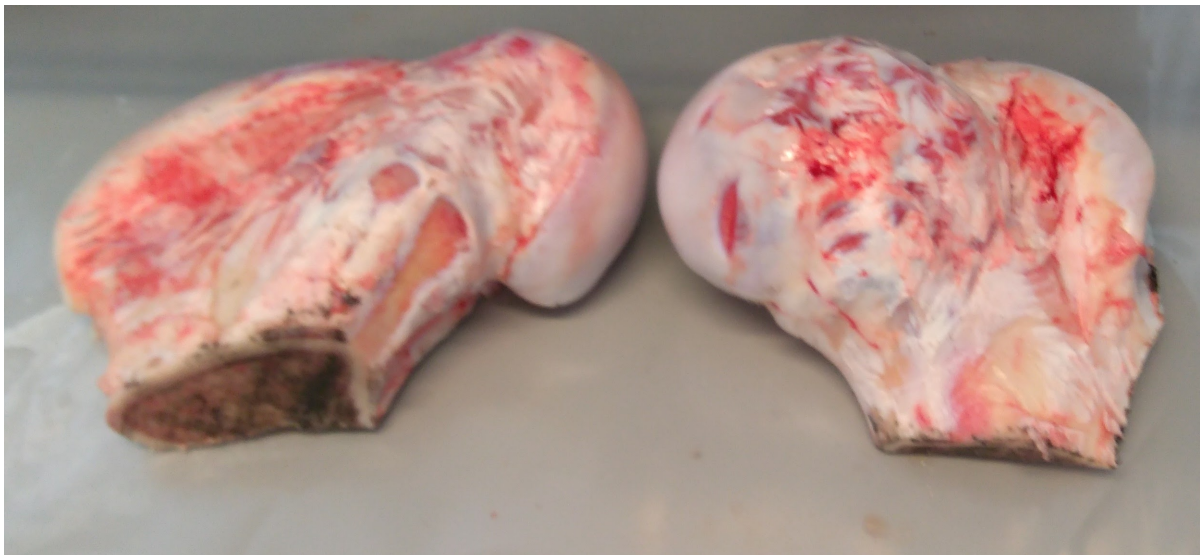


Figure 16. Bovine femoral condyles divided by a sagittal cut.

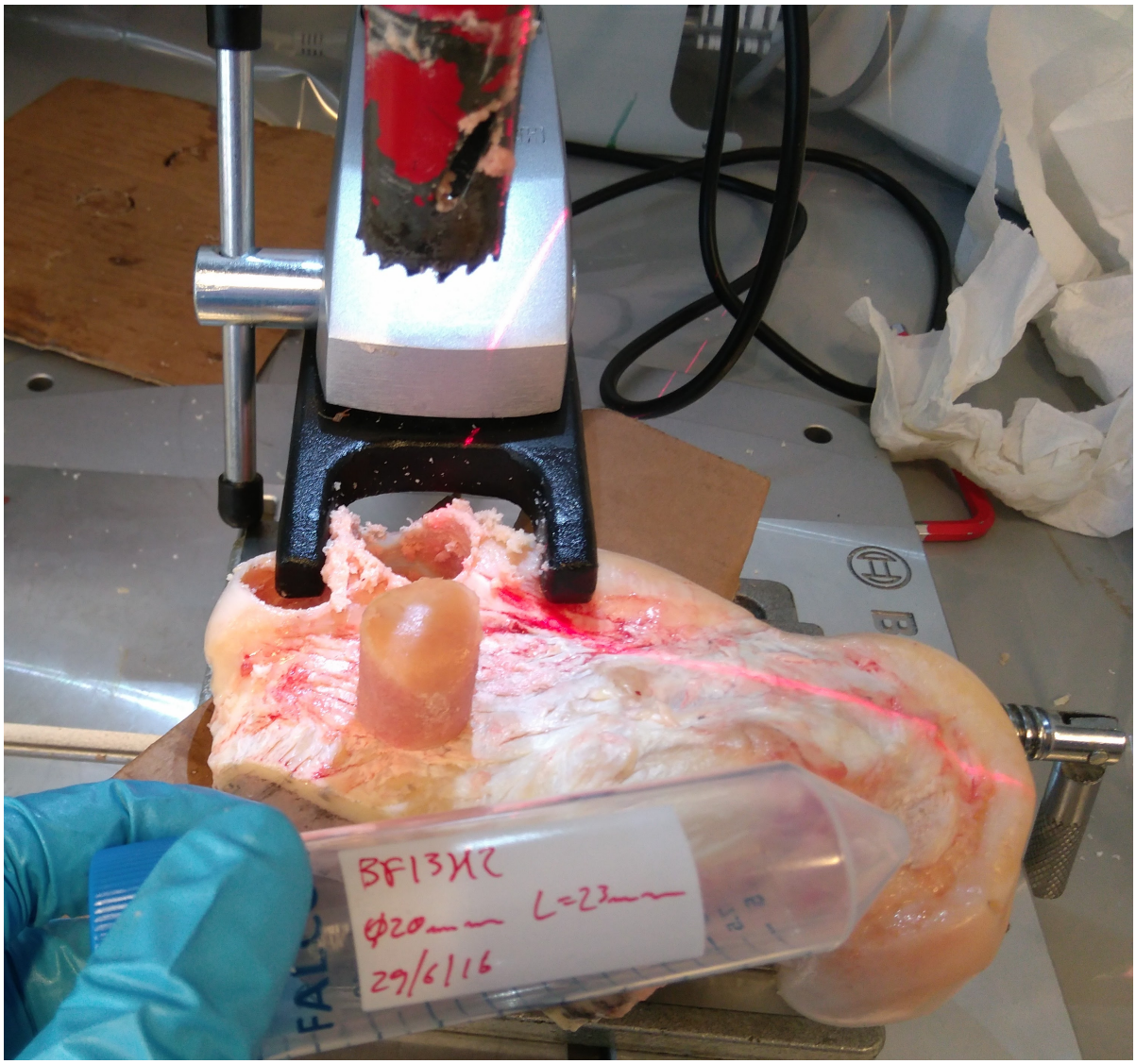


Figure 17. Bovine cylinder sample extraction.

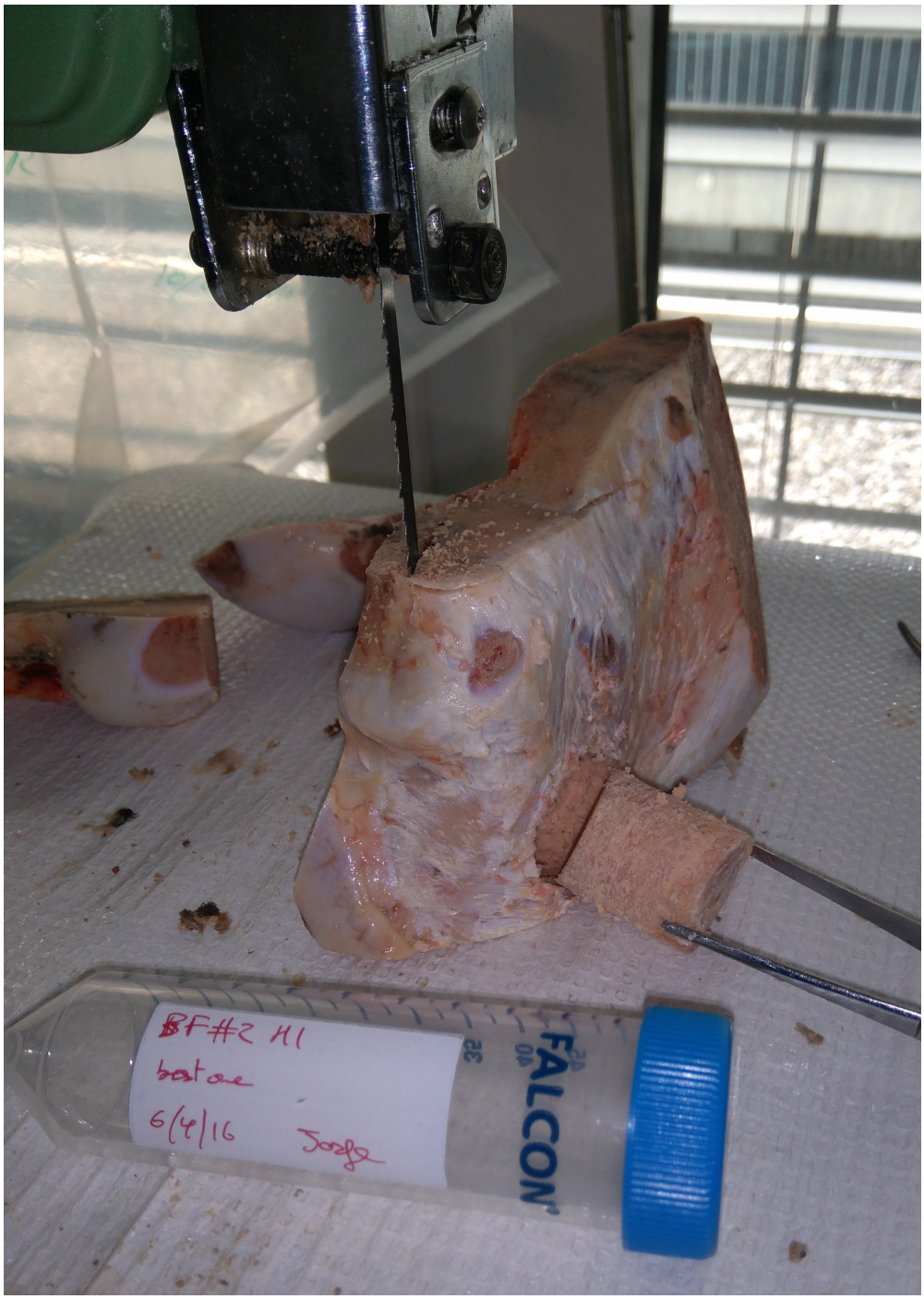


Figure 18. Bovine sample extraction. Example of cylinder cutting for extraction.

B.II. Hydrogel Recipes

The hydrogel preparation protocols followed in this work were inspired by (Zuidema et al. 2014).

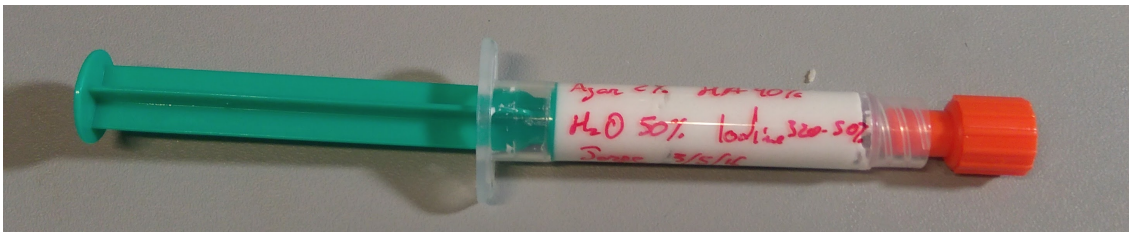


Figure 19. Example of hydrogel ready to be stored at 4 °C.

B.II.1. Agar-Agar

Protocol to prepare 2 % Agarose based viscous solution

Materials

- Microwave (or other heating device able to boil water)
- Balance with accuracy to mg
- Vortex mixing device
- 200 mg of Agar-Agar (Ref: 05039, Sigma-Aldrich)
- 1 Falcon tube 50ml
- 1 support for Falcon tube
- 10 ml of solution
- Option1- H_20: Bi-distilled water
- Option2- Saline solution: Bi-distilled water with PBS
- Option3- 50 % of any of precedent options + 50 % Iodine (Hexabrix 320)
- 2 syringes 10 ml
- 1 lab spoons
- 1 lab spatula
- 1 lab tray with ice
- 1 pipette 10 ml
- 1 extra syringe 10 ml
- 1 thermometer

Conditions

- The whole procedure has been done and developed in the Laboratory of Biomechanical Orthopaedics EPFL, Station 15, 1015 Lausanne, Switzerland.
- Room temperature was between 18-25 °C.
- No assistance required.

Procedure

1. Place the Falcon tube in the support and open it.
2. Measure 10 ml of solution with the pipette and put it into the Falcon tube.
3. Heat the new solution slightly above 80 °C (85 to 95 °C ideally).
4. Weight 200 mg of Agar-Agar.
5. Transfer the 200 mg of Agar-Agar in the Falcon tube, close it and mix it with the Vortex.
6. Open the Falcon tube again and heat the solution with a microwave or other heating device. The solution need to reach 95 °C to activate the Agar-Agar[1]. If you don't have a thermometer available, you can close the Falcon tube after heating and cool it down quickly with ice. Then the solution should become gelatine like at room temperature. If not, it means that the solution did not reach the activation temperature of Agar-Agar, so it must be heated again.
7. Break the gelatine created using a lab spatula and fill a 10 ml syringe with it.
8. Void the filled syringe into another 10 ml syringe to continue breaking the gelatine peaces into smaller particles. Repeat at least 3 times.
9. Close the syringe and write on it the composition of the solution (2 % Agar) and date.
10. Store it at 4° C.

[1] *Agar-Agar* ***05039 Sigma-Aldrich*** *properties*: grade - for microbiology; ign. residue ≤5 %; loss ≤12 % loss on drying; pH 6-7.5 (1.5 % after autoclaving); mp ≈ 85-95 °C; transition temp. gel point 33-37 °C (1.5 % solution, after autoclaving); gel strength >800 g/cm^2 (1.5 % gel, Nikkan, after autoclaving); solubility – H_2O: soluble 1.5 % at 70 °C, clear to very faintly turbid, almost colourless.
http://www.sigmaaldrich.com/catalog/product/sial/05039?lang=fr&region=CH&cm_sp=Insite-_-prodRecCold_xorders-_-prodRecCold2-1

B.II.1. Agar-Agar + HaP

Protocol to prepare 2 % Agarose based viscous solution with 40 % of Hydroxyapatite

Materials

- Microwave (or other heating device)
- Balance with accuracy to mg
- Vortex mixing device
- 200 mg of Agar-Agar (Ref: **05039, Sigma-Aldrich)**
- 4 g of Hydroxyapatite (HA) (Ref: **04238, Sigma-Aldrich**)
- 1 Falcon tube 50ml
- 1 support for Falcon tube
- 10 ml of solution
- Option1- H_20: Bi-distilled water
- Option2- Saline solution: Bi-distilled water with PBS
- Option3- 50 % of any of precedent options + 50 % Iodine (Hexabrix 320)
- 2 syringes 10 ml
- 2 lab spoons (one for the Agar-Agar and other for the HaP)
- 1 lab spatula
- 1 lab tray with ice
- 1 pipette 10 ml
- 1 extra syringe 10 ml
- 1 thermometer

Conditions

- The whole procedure has been done and developed in the Laboratory of Biomechanical Orthopaedics EPFL, Station 15, 1015 Lausanne, Switzerland.
- Room temperature was between 18-25 °C.
- No assistance required.

Procedure

1. Place the Falcon tube in the support and open it.
2. Measure 10 ml of solution with the pipette and put it into the Falcon tube.
3. Weight the 4 g of HaP and add them into the Falcon tube.
4. Close and mix with the aid of the Vortex mixing device.
5. Heat the new solution slightly above 80 °C (85 to 95 °C ideally).
6. Weight 200 mg of Agar-Agar.
7. Transfer the 200 mg of Agar-Agar in the Falcon tube, close it and mix it with the Vortex.
8. Open the Falcon tube again and heat the solution with a microwave or other heating device. The solution need to reach 95 °C to activate the Agar-Agar[1]. If you don't have a thermometer available, you can close the Falcon tube after heating and cool it down quickly with ice. Then the solution should become gelatine like at room temperature. If not, it means that the solution did not reach the activation temperature of Agar-Agar, so it must be heated again.
9. Break the gelatine created with HaP using a lab spatula and fill a 10 ml syringe with it.
10. Void the filled syringe into another 10 ml syringe to continue breaking the gelatine peaces into smaller particles. Repeat at least 3 times.
11. Close the syringe and write on it the composition of the solution (2 % Agar + 40 % HaP) and date.
12. Store it at 4° C.

[1] *Agar-Agar* ***05039 Sigma-Aldrich*** *properties*: grade - for microbiology; ign. residue ≤5 %; loss ≤12 % loss on drying; pH 6-7.5 (1.5 % after autoclaving); mp ≈ 85-95 °C; transition temp. gel point 33-37 °C (1.5 % solution, after autoclaving); gel strength >800 g/cm^2 (1.5 % gel, Nikkan, after autoclaving); solubility – H_2O: soluble 1.5 % at 70 °C, clear to very faintly turbid, almost colourless.
http://www.sigmaaldrich.com/catalog/product/sial/05039?lang=fr&region=CH&cm_sp=Insite-_-prodRecCold_xorders-_-prodRecCold2-1

B.II.2. Hyalectine

Protocol to prepare 3.5 mg/ml Hyaluronic Acid based viscous solution with 40 % of Hydroxyapatite

Materials

- Balance with accuracy to µg
- 7 mg of Hyaluronic Acid (Hy) ("HYALECTIN", 811261, 800KD, Fidia Farmaceutici s.p.a., Padova, Italy**)**
- Polystyrene antistatic weighing (PAW) dishes
- 2 ml of solution
  - Option1- H_20: Bi-distilled water
  - Option2- Saline solution: Bi-distilled water with PBS
  - Option3- 50 % of any of precedent options + 50 % Iodine (Hexabrix 320)
- Syringes 3 ml
- 2 lab spoons (for weighing, one for the Agar-Agar and other for the HaP)
- 1 lab spatula (very small, as it is to mix a 2 ml solution)

Conditions

- The whole procedure has been done and developed in the Laboratory of Biomechanical Orthopaedics EPFL, Station 15, 1015 Lausanne, Switzerland.
- Room temperature was between 18-25 °C.
- No assistance required.

Procedure

1. Weight the 7 mg of Hy.
2. Measure the 2 ml of solution and add them carefully to the PAW dish containing the Hy.
3. Use the lab spatula to mix powder within the solution until complete dissolution of the Hyalectine powder.
4. Keep removing until getting a homogeny gel like.
5. Leave the gel repose for 10 mins at room temperature.
6. Clean the lab spatula.
7. Use the lab spatula to place the gel into a 3 ml syringe.
8. Close the syringe and write on it the composition of the solution (35 mg/ml Hy) and date.
9. Store it at 4° C.

B.II.3. Hyalectine + HaP

Protocol to prepare 3.5 mg/ml Hyaluronic Acid based viscous solution with 40 % of Hydroxyapatite

Materials

- Balance with accuracy to µg
- 7 mg of Hyaluronic Acid (Hy) ("HYALECTIN", 811261, 800KD, Fidia Farmaceutici s.p.a., Padova, Italy**)**
- 800 mg of Hydroxyapatite (HA) (Ref: **04238, Sigma-Aldrich**)
- Polystyrene antistatic weighing (PAW) dishes
- 2 ml of solution
  - Option1- H_20: Bi-distilled water
  - Option2- Saline solution: Bi-distilled water with PBS
  - Option3- 50 % of any of precedent options + 50 % Iodine (Hexabrix 320)
- Syringes 3 ml
- 2 lab spoons (for weighing, one for the Agar-Agar and other for the HaP)
- 1 lab spatula (very small, as it is to mix a 2 ml solution)

Conditions

- The whole procedure has been done and developed in the Laboratory of Biomechanical Orthopaedics EPFL, Station 15, 1015 Lausanne, Switzerland.
- Room temperature was between 18-25 °C.
- No assistance required.

Procedure

1. Weight the 7 mg of Hyalectine (Hy).
2. Weight the 800 mg of HaP.
3. Mix both powders in a polystyrene antistatic weighing dish with the help of a tiny lab spatula.
4. Measure the 2 ml of solution and add them carefully to the PAW dish.
5. Use the lab spatula to mix powders within the solution until complete dissolution of the powder.
6. Keep removing until getting a homogeny gel like.
7. Leave the gel repose for 10 mins at room temperature.
8. Clean the lab spatula.
9. Use the lab spatula to place the gel into a 3 ml syringe.
10. Close the syringe and write on it the composition of the solution (35 mg/ml Hy + 40 % HaP) and date.
11. Store it at 4° C.

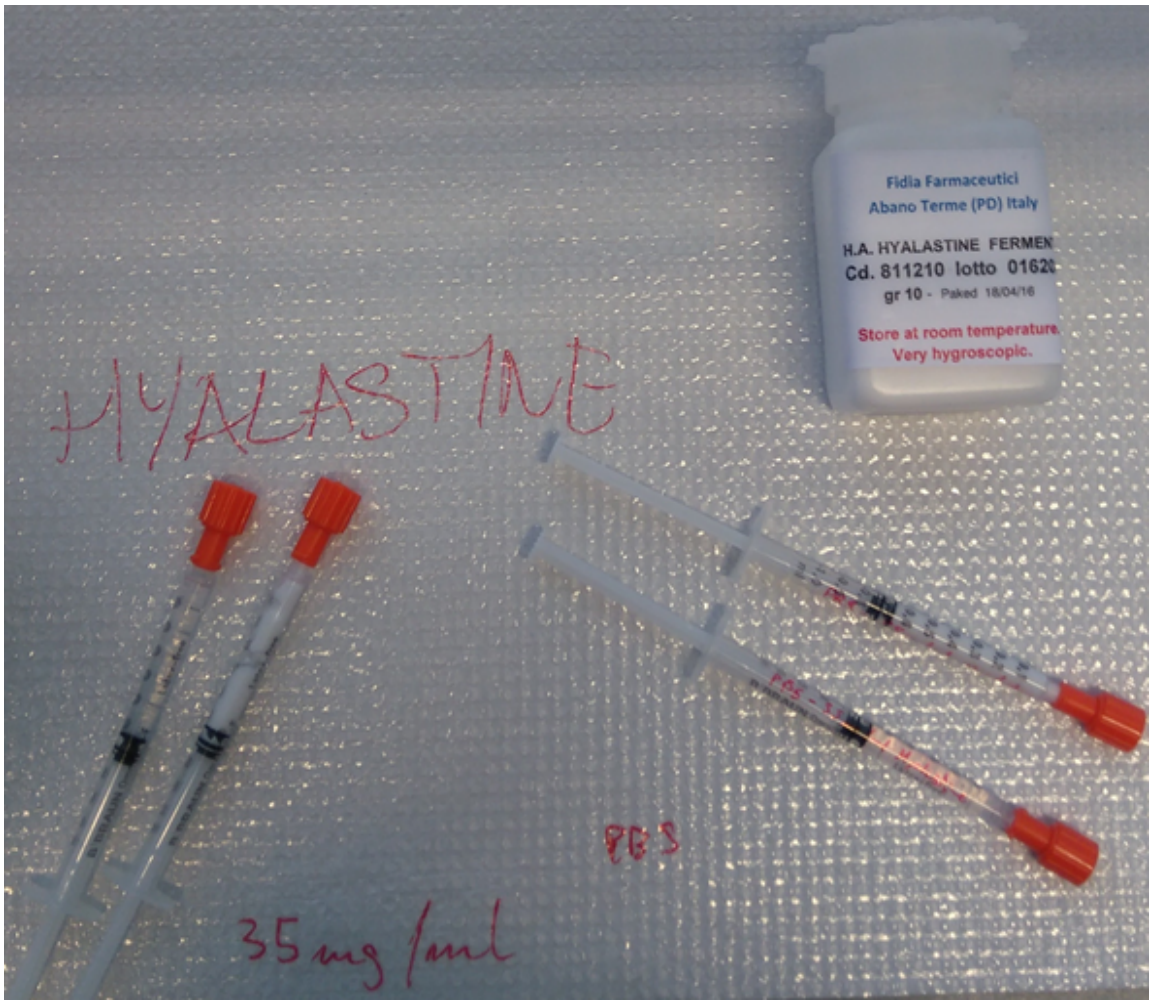


Figure 20. Example of hydrogel preparation with Hyaluronic Acid and Hydroxyapatite.

B.III. µCT Scans

B.III.1. Scan Procedure per sample

Materials

- µCT (Skyscan 1076)
    - Keep record of calibration
- Scanning fixture (Al + Sagex tubes)

Conditions

Specimens are in plastic tubes at still frozen (from -20 °C storage)

The whole procedure will be done at EPFL LBO

Procedure

Preparation

1. Turn on the µCT machine and the software (SkyScan1076 InVivo CT)
2. Start Source and wait 15 min
3. Press “Remove Object”
4. Open the µCT

Mounting Procedure

5. Assemble the fixture (Al support tubes + Sagex) in the µ-CT
6. Place specimen tube on the Sagex that it is not interfering with the Al support tube and it is centred properly
7. Close the machine
8. Click scout scan

Setting / Flat field correction (only the first time of each group)

9. Select Menus/Options/Filter = 1 mm Al
10. Enter the scanner parameter: Menus/Options/XraySource= Voltage 100 kV, Current 100 mA
11. Enter Menus/Options/Acquisition parameter: Scanning width = 35 mm,
Pixel size = 35 um, Rotation step = 0.5, unselect “Use 360 deg. Rotation”,
Averaging = 2, Exposure time = 120 ms then CLOSE
12. Press simultaneously CTRL+ALT+SHIFT+S
13. Menus/Options/Scanning modes/ and control parameters (point 3). Then check “acquire single…” and press OK
14. Check position by clicking the “camera icon”

Scanning (7 minutes)

1. Centre specimen by double-clicking on the scout scan
2. Menus/Options/Acquisition
3. Enter File name “**specimen_name**”
4. Browse directory
5. Select “Start Scan” (about 6 minutes)
6. Select “Remove Object ”
7. Put the specimen tube back into the freezer -20 °C

Reconstruction

1. Start “NRecon” software
2. Open dataset by selecting one image
3. Select reconstruction range
4. Select Parameters: check “Smoothening=1”, ”Ring artefact reduction”=20, “Beam-hardening” = 50%
5. Select Output option: 0-0.04, file format = TIF
6. Add to batch
7. Start batch manager after last specimen is in the queue

B.III.3. Bone Density Parameters

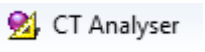
Materials

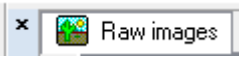
- PC with CT Analyser
- Reconstructed files

Conditions

- Done in the Laboratory of Biomechanical Orthopaedics EPFL, Station 15, 1015 Lausanne, Switzerland

Procedure

25. Open CT Analyser 

26. Click Open, browse into your reconstruction file's folder (normally finished in "_rec") and double click in one of the reconstructed slices.
27. Use the window "Raw images" 

to select the region of interest.
28. Click in "Regions of interest preview"  and save the region selected ()
29. Click in "Binary selection preview" 
30. Click in the dataset tab from the Histogram's window 

31. Then go to the sub-tab called "Bone mineral density" and note the value next to "Mean (value):". this is the mean BMD of the selected region of interest.
32. Find 3D analysis tools () in the morphometry preview ()
33. Use the batch tool () from the custom processing view () to perform many measurements in one row and get BMD and other parameters saved in a text file.

B.V. Screw Hole Drilling

Materials

- Column drill machine (Bosch PBD 40, Leinfelden-Echterdingen, Germany)
- Metal drill bit (4 mm outer diameter)
- Vice (prefereably one that can be fixed to the column drill work plate)
- Protection equipment (mask, security glasses, dissection lab coat, lab globes)
- Dissection kit
- Surfaces protection paper
- Bovine bone cylindrical samples
- Caliper
- 1 studio tables
- Bowl or other appropriate recipient for 1 L of warm water

Conditions

- The whole procedure was done in the P2 Lab of LBO EPFL, 1015 Lausanne, Switzerland
- Room temperature

Procedure

Specimen preparation

1. The bovine bone cylindrical samples had been stored at -20° inside a lab tube.
2. The bovine samples were prewarmed sumerging the closed lab tubes in a warm bath of water (idealy 35 to 40 °C).

Column Drill preparation

3. Switch on the drilling machine.
4. Set drilling speed at 400 rpm.
5. Switch off the drilling machine.
6. Switch the Display mode to “Depth”.
7. Fix the drill bit.
8. Switch on the light and the laser assistant for centring
9. Protect the working plate with some protecting paper to avoid extra cleaning
10. Place and fasten the vice.

Drilling procedure

11. Place the cylindrical sample in the vice trying to keep the cylindrical axis aligned with the drilling axis. Small tilt might be allowed to assure cortical surface perpendicular to the drilling axis.
12. Close the vice.
13. Set the drill machine initial position at a few millimetres from the cortical surface and fasten.
14. Use the wheel of the drilling machine to approximate the drill bit to the cortical surface of the sample and set the “zero position” of the drill machine by pushing the button “set zero”.
15. Leave the drilling machine go back to initial position.
16. Switch on the drilling machine.
17. Use the wheel of the drilling machine to approximate the drill bit to the cortical surface and continue drilling at slow speed until the display shows the desired depth.
18. Leave the drilling machine go back to initial position.
19. Unfasten the vice and continue with next samples since step 11.
20. Switch off the drilling machine.

B.VI. Augmentation biomaterial Injection

Materials

- Augmentation viscous solution in container syringe
- Cylindrical bovine sample
- Special syringe DISTRITIP 1250 µL (DISTRITIP 1250, GILSON, France)
- Lab globes
- Paper towels

Conditions

- The whole procedure has been done and developed in the Laboratory of Biomechanical Orthopaedics EPFL, Station 15, 1015 Lausanne, Switzerland.
- Room temperature was between 18-25 °C.
- No assistance required.
- The hydrogel have been previously prepared and stored at 4 °C for no more than 10 days.
- The bovine samples have been previously warmed up to room temperature and the hole to be filled has already been drilled.

Procedure

1. Take the hydrogel container syringe from the 4 °C storage and open it.
2. Remove the plunger of the DISTRITIP syringe.
3. Use the hydrogel containing syringe to fill the DISTRITIP by its back.
4. Close syringes that contain the remaining hydrogel and store it at 4 °C.
5. Hold the sample with one hand. Use paper towels to avoid slipping.
6. Place the needle like part of the DISTRITIP inside the sample hole as deep as possible taking care to don't damage the bone sample.
7. Push the plunger carefully and remove the syringe slowly when feeling resistance.
8. Continue filling the sample hole until some gel overflows.
9. Continue from step 6 filling the rest of the samples until the DISTRITIP get empty.

Important notes

10. Avoid applying too much pressure when filling the sample hole, otherwise the solution could damage the sample and fill other spaces.
11. Continue with the screw insertion procedure without storing the samples for long time to avoid gel dehydration.

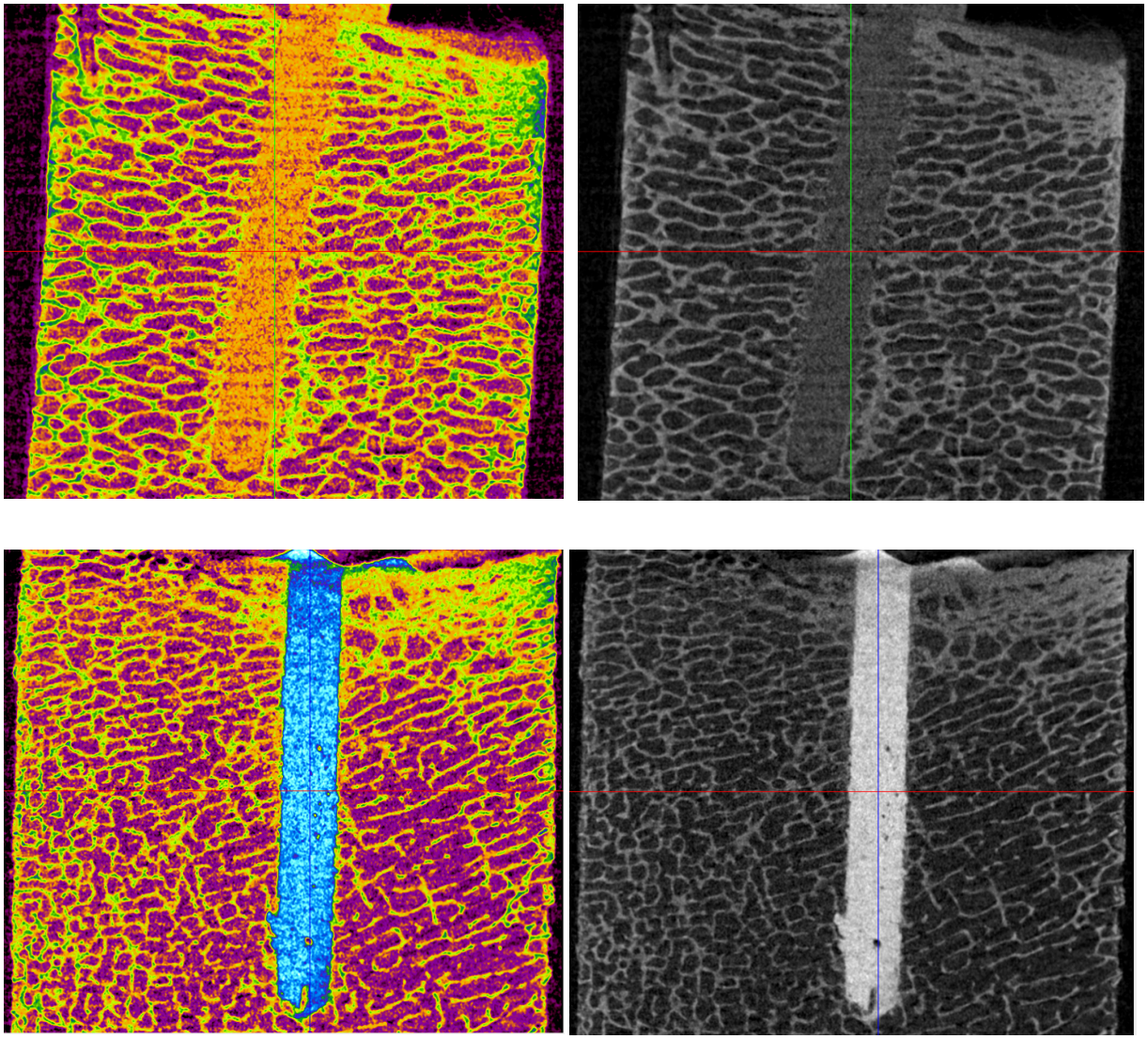


Figure 22. µCT scans to show that the injection technique is good enough to avoid the formation of big air bubbles.

Figure 21. Example of sample just filled with HyHaP viscous solution.

B.VII. Screw Insertion

Materials

- Cylindrical bovine sample
- 4 mm diameter x 26 mm length cancellous bone screws (ref: 604026, Cancellous Screw, Stryker, Selzach, Switzerland)
- Screwdriver (Stryker, Selzach, Switzerland)
- Calliper
- Lab globes
- Paper towels

Conditions

- The whole procedure has been done and developed in the Laboratory of Biomechanical Orthopaedics EPFL, Station 15, 1015 Lausanne, Switzerland.
- Room temperature was between 18-25 °C.
- No assistance required.
- The bovine samples have been previously warmed up to room temperature and the screw hole has already been drilled and eventually filled with a viscous solution.

Procedure

1. Fit the screwdriver into the screw head.
2. **Hold the sample with one** hand. Use paper towels to avoid slipping.
3. **Place the** cancellous screw carefully in the **sample hole** and apply torque with the screw driver**.**
4. Eventually stop screwing to measure the depth**.** Use the calliper to measure the distance between the cortical surface and the top of the screw head.
5. Continue screwing and keep measuring till the desired depth. For 18 mm screw depth, the distance from cortical surface to the top of the screw head should be 8 mm (for 26 length screws).

Important notes

6. **Continue with the** moulding and pull-out **procedure without storing the samples for lon**g time to avoid gel dehydration and bone degradation.

Figure 24. Example of screw insertion in bovine bone cylindrical samples.

B.VIII. Sample Holder Moulding

Materials

- Cylindrical
- Bovine samples
- Aluminium cylindrical moulding pieces
- Screw holding device (Stryker, Selzach, Switzerland)
- Fast curing methyl methacrylate-based resin:
    - Yellow powder (64708806, Technovit 3040, Kulzer, Germany)
    - Universal liquid (66022678, Technovit Universal liquid, Kulzer, Germany)
- Disposable glasses
- Chemical lab protection kit: mask, security glasses, lab globes, appropriate lab coat
- Gas extraction hood
- 50 ml syringes
- Syringe caps
- 2.5 ml disposable Pasteur pipettes
- 1 lab spoon
- Disposable lab spatulas
- Balance with accuracy to mg
- Lab protecting paper
- Paper towels
- Scissors

Conditions

- The whole procedure has been done and developed in the Laboratory of Biomechanical Orthopaedics EPFL, Station 15, 1015 Lausanne, Switzerland.
- The whole procedure must be done under a gas extraction hood.
- Room temperature was between 18-25 °C.
- No assistance required.
- The bovine samples have been previously warmed up to room temperature and the screw hole has already been drilled and eventually filled with hydrogel.
- The following procedure was developed to mould 2 bone samples, which was optimized considering the fast hardening of the resin used. If the operator is fast enough up to 4 samples can be moulded at the same time.

Procedure

1. Cover the working space under the extraction hood with appropriate surfaces protection paper or paper towels.
2. Check that the bone sample length is no longer than the internal high of the moulding pieces.
3. Using the screw holding device to hold one screw inserted in a bone sample and mount the moulding pieces as shown in the picture:

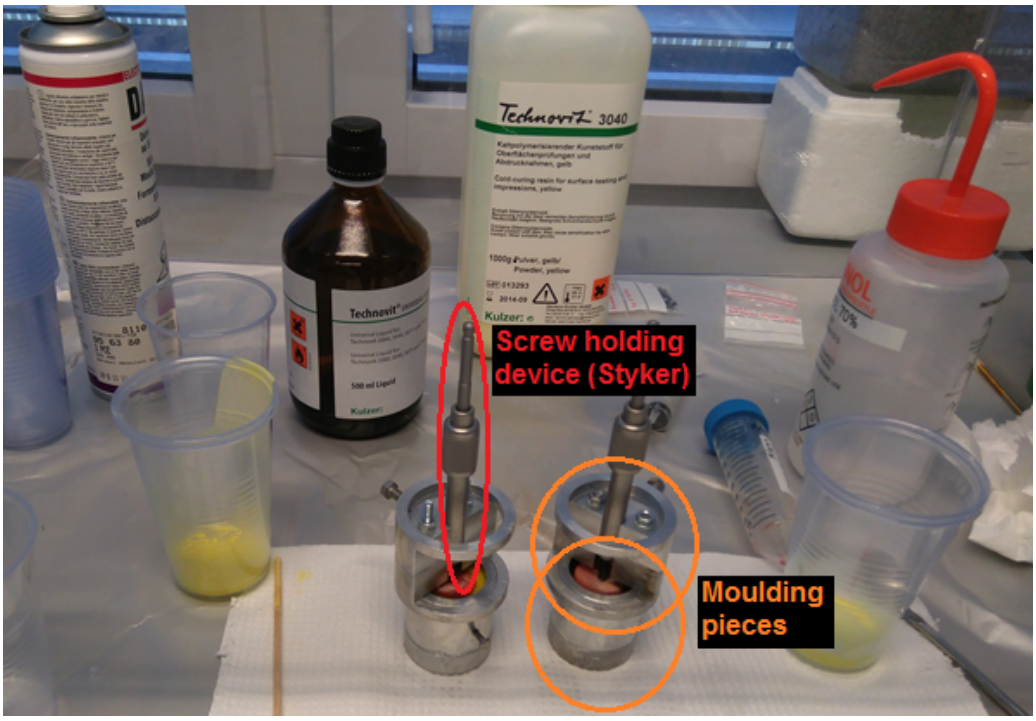


Figure 25. Example of moulding setup.

4. Put labels to the moulds with appropriate ID information of the samples.
5. **Weight** 23 g (for 2 to 3 samples) of Technovit 3040 power in a disposable glass.
6. Weight 17 g (for 2 to 3 samples) of Universal liquid in another disposable glass.
7. Remove the plunger of a 50 ml Syringe and close it with appropriate syringe cap, and place the syringe in a disposable glass with the closed tip in the bottom and the open back up.
8. Cut the needle like part of 2 disposable Pasteur pipettes like shown in the picture:

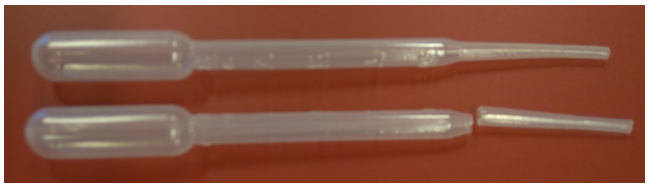


Figure 26. Example of needle like cutting.

9. Save the needle like part and discard the rest of the disposable Pasteur pipette.
10. Dump the 34 g of Universal liquid in the disposal glass that contain the 46 g of Technovit 3040 and immediately start mixing with a disposable spatula.
11. Keep removing vigorously for no longer than 1 minute.
12. Dump the mixed liquid into the 50 ml syringe.
13. Close the back of the 50 ml syringe with the plunger, turn it carefully to have the syringe tip looking up and remove the cap.
14. Push the plunger to remove air from the syringe.
15. Put one of the cut needle like part of the disposable Pasteur pipette on the 50 ml syringe tip, as it was a needle.
16. Insert the needle like between bone and mould walls and start injecting the resin.

17. Keep injecting until the resin fills the mould but stop before the resin overflows on the cortical surface of the bone. IMPORTANT: avoid any resin over the cortical surface, otherwise the pull-out test will be influenced by an augmented cortical shell.
18. Quick repeat from step 15 with the second sample, before the resin become too viscous and hence less injectable.
19. If needed replace the needle like part by the second one previously prepared.
20. When finished, discard properly every disposable material.
21. Leave the moulds under the extraction hood for 30 mins before performing pull-out test.

Important notes

22. **Continue with** pull-out **procedure without storing the samples for lon**g time to avoid gel dehydration and bone degradation.
23. Clean the moulding pieces with ethanol, dry properly and lubricate before reuse.

Figure 27. Example of moulding procedure.

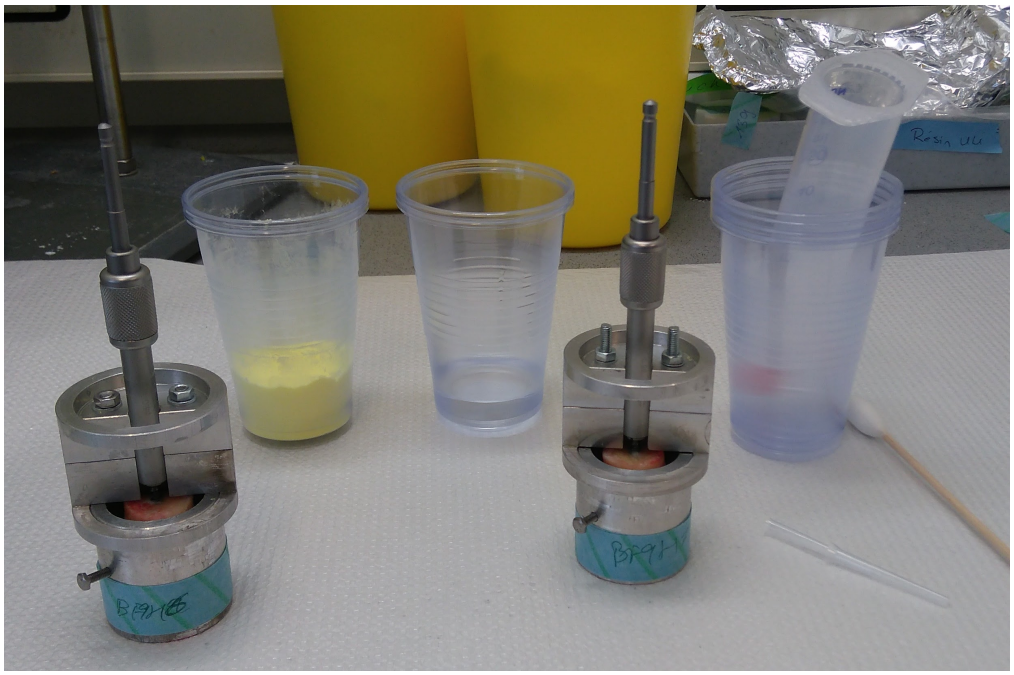


Figure 28. Example of mould labelling.

B.IX. Pull-Out Test

Materials

- Traction Machine (Instron 3000, Massachusetts, USA)
- Bone samples moulded in polymerized resin
- Pull-out mechanical setup
- Mechanical lab protection kit: security glasses, lab globes, appropriate lab coat
- Paper towels
- Hex key set

Conditions

- The whole procedure has been done and developed in the Laboratory of Biomechanical Orthopaedics EPFL, Station 15, 1015 Lausanne, Switzerland.
- The whole procedure must be done under a gas extraction hood.
- Room temperature was between 18-25 °C.
- Assistance required from the person responsible of the machine.

Procedure

Preparation

1. Switch of the Instron 3000 (at least 5 minutes of preheating time are necessary).
2. Switch on the PC controlling Instron 3000.
3. Open Wave Console and WaveMatrix.

Mechanical setup

4. Fit the 150 mm diameter black steel plate to the bottom of the 2 kN Instron sensor. Use 6 x M6 screws.
5. Fit the plate + Instron sensor assembly to the Instron 3000 base plate. Use the 6 x M8 screw holes available. IMPORTANT: to assure the centring of those pieces, use the 40 mm diameter aluminium ring. This ring must be between both black steel plates. The ring fits in the base Instron 3000 base as well as in the bottom part of the plate + sensor assembly.
6. On the top of the sensor (sensitive part) another aluminium ring must be placed to avoid direct contact with the rest of the mechanical parts. This aluminium ring of 15 mm of diameter fits in the centre of the top side of the sensor.
7. Take the “samples holding assembly” and fit a headless M6x50mm screw in the centred bottom screw hole. Let the headless screw get into the hole until the end. 30 mm of headless screw should remain outside the assembly piece. Insert that screw through the “fasten device” (see picture) into the screw hole on the centre of the top of the 2 kN sensor.

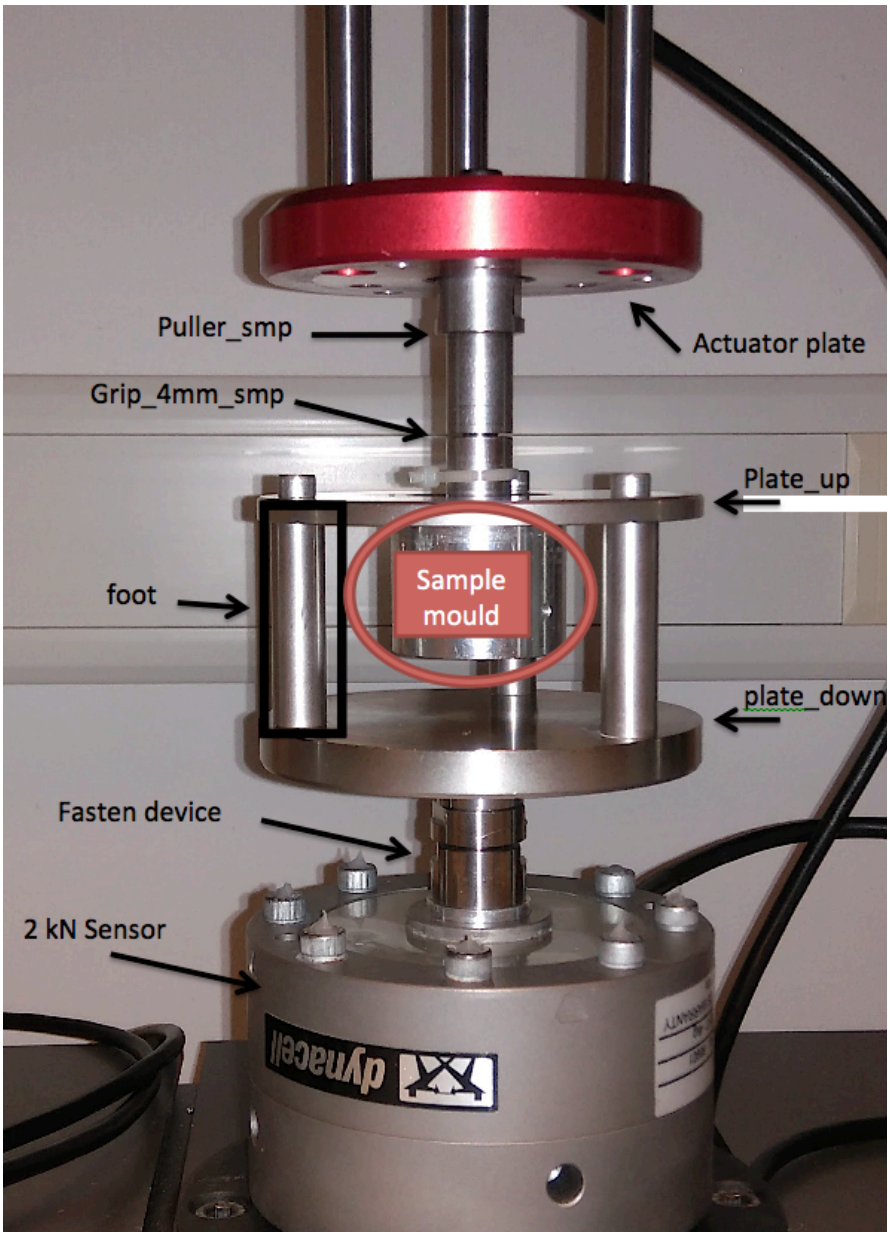
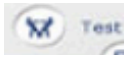


Figure 29. Example of pull-out mechanical setup.

8. Fit the “puller_smp” to the “actuator_plate”
9. Insert the sample with mould as holder, through the space between foots, with the screw head up.
10. Use the manual control of the actuator to approach the “puller_smp” to the screw head. Use the “grip_4mm_smp” to clamp the screw to the “puller_smp”.

PC software preparation

11. Click in Method  and load an appropriate method for pull-out test (ask assistance from the responsible person).
12. Click in Test  or  and charge the convenient pull-out test. If needed, define the test as a continuous movement of traction at 5 mm per minute.
13. Calibrate the sensor.
14. Set appropriate load and position limits.
15. Move the actuator up with the manual control until the bone sample mould touch the "plate_up".
16. Set digital position to 0.
17. Chose the name of the sample.
18. Start the test.
19. When the test finishes note the maximum pull-out strength and save the Excel files to a convenient folder.
20. Move manually the actuator up. Remove the extracted screw from the Grip_4mm_smp and clean it for further use.
21. Continue from step 15 for remaining samples.

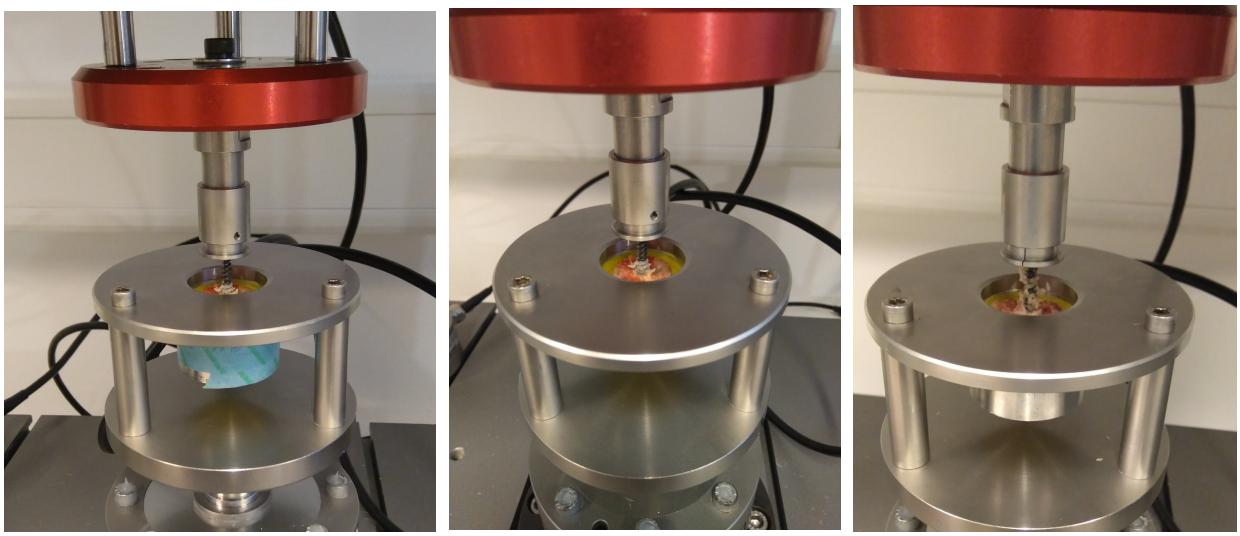


Figure 30. Example of extraction secuence.

C. Statistics

Table 6. Statistical comparison of the 4 bovine groups with biomaterial and the Control as reference. Analysed with Matlab functions: *anova1* and *multcompare*.

Agar	Hyalectine	Agar_HaP	Hyalectine_HaP
0.696	0.4833	0.00063	0.00011

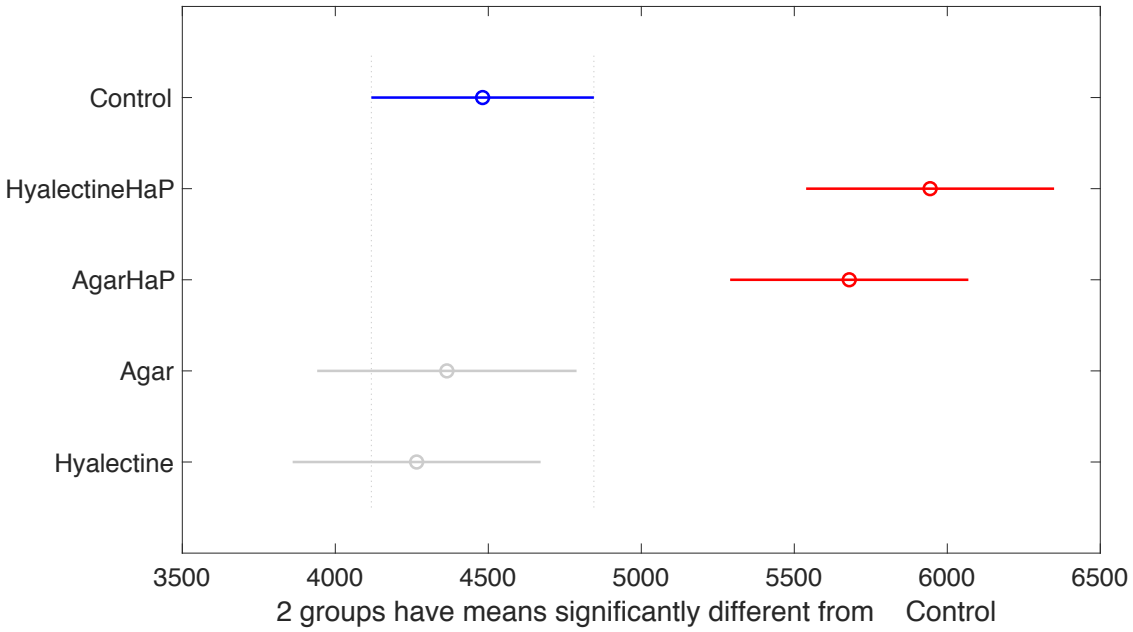


Figure 31. Multiple comparison of group means (Control)

D. Samples Data

Table 7. BMD values and PO(N) forces of samples from bovine group Control.

#15 samples	CONTROL group	
	BMD (g/cc)	FORCE (N)
BF6H1	0.164	626.4
BF6H2	0.0923	489.2
BF6H3	0.16582	638.4
BF6H4	0.14613	956.2
BF6H5	0.35378	1449
BF6H7	0.17984	843.5
BF8H8	0.15	566.64
BF8H9	0.14	439.21
BF9H6	0.17293	600
BF9H7	0.25623	1370
BF10H2	0.26441	1080
BF12H1	0.32207	1850
BF12H2	0.31319	1425
BF12H9	0.24922	1000
BF13H3	0.26884	1170
Mean	0.215917	966.9033
STD	0.078485	420.9208

Table 8. BMD values and PO(N) forces of samples from bovine group HyHaP.

#12 samples	HyHaP group	
	BMD (g/cc)	FORCE (N)
BF10H6	0.27734	1777.8
BF11H7	0.22678	1158.2
BF11H8	0.295	1705.1
BF11H9	0.22838	1109.4
BF12H6	0.15898	895.4
BF12H8	0.11402	686.7
BF12H10	0.16748	1046.2
BF13H1	0.13492	884.9
BF13H2	0.13086	787.7
BF13H5	0.11205	840.1
BF11H1	0.3332	1735.4
BF11H2	0.3121	1870.6
Mean	*0.20759*	*1208.13*
STD	*0.08148*	*438.30*

Table 9. BMD values and PO(N) forces of samples from bovine group AgarHaP.

#9 samples	AgarHaP group	
	BMD (g/cc)	FORCE (N)
BF7H3	0.09477	568.52
BF7H4	0.19984	927.77
BF7H5	0.10964	683.97
BF7H6	0.0908	460
BF7H7	0.11066	710.46
BF7H8	0.14703	947.03
BF9H9	0.21357	1230
BF10H1	0.19381	880
BF10H4	0.1753	870
BF9H3	0.351	1886.7
BF9H4	0.3067	1952.4
BF9H8	0.2857	1857.6
BF10H3	0.3327	1799.9
Mean	*0.20089*	*1136.49*
STD	*0.09216*	*546.21*

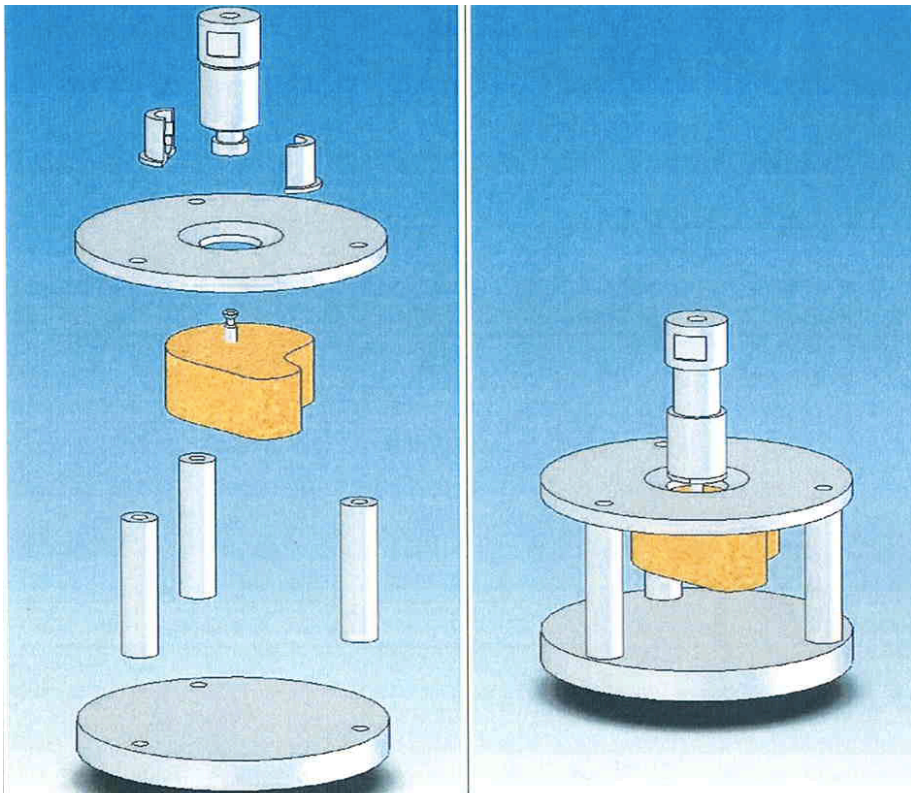
Table 10. BMD values and PO(N) forces of samples from bovine group Agar.

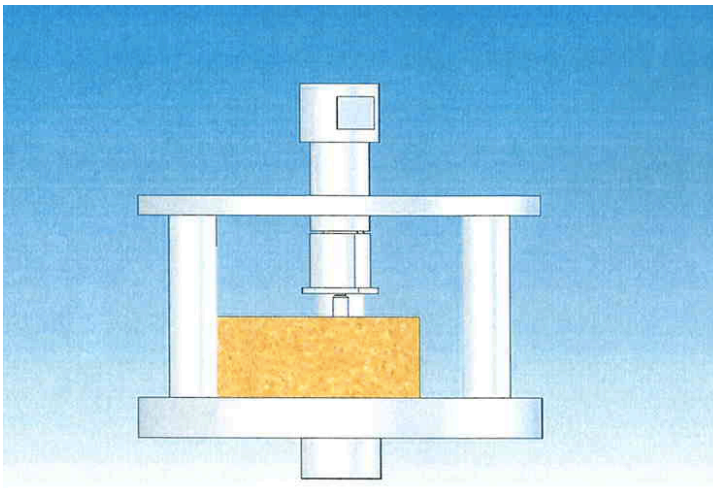
#11 samples	Agar group	
	BMD (g/cc)	FORCE (N)
BF8H4	0.175	770
BF8H5	0.249	1150
BF8H6	0.2386	1200
BF8H7	0.136	520
BF14H2	0.19181	747.679
BF14H3	0.21939	903.6843
BF15H4	0.10437	476.5975
BF15H6	0.15609	670.3511
BF16H2	0.29957	1174.3051
BF16H3	0.11127	516.1045
BF16H4	0.27364	1043.8986
Mean	*0.19589*	*833.87*
STD	*0.06569*	*276.45*

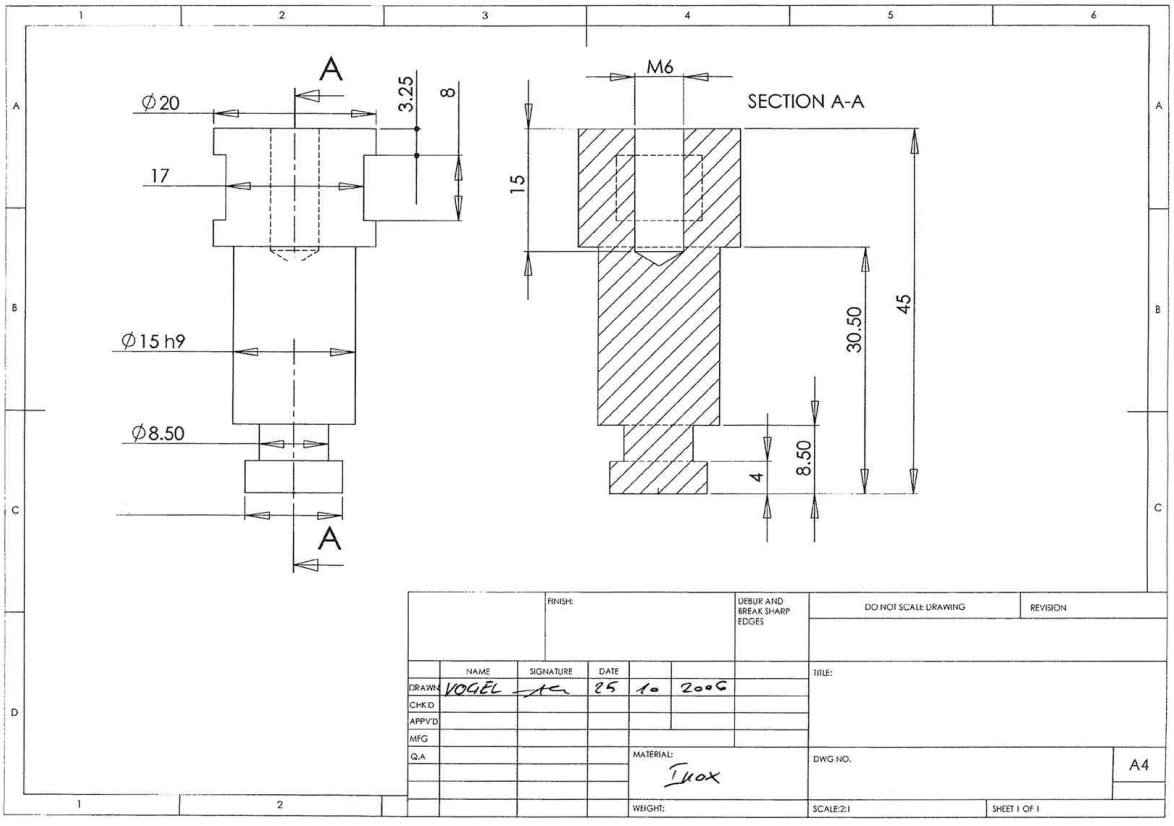
Table 11. BMD values and PO(N) forces of samples from bovine group Hyalectine

#11 samples	Agar group	
	BMD (g/cc)	FORCE (N)
BF14H1	0.07685	436.2012
BF14H4	0.28451	1276.1796
BF14H6	0.13871	545.4111
BF14H7	0.15039	708.9078
BF14H8	0.21918	883.047
BF14H10	0.24037	912.421
BF15H1	0.30918	1483.4686
BF15H2	0.19955	854.924
BF15H3	0.23763	934.7323
BF15H5	0.09493	415.7593
BF15H7	0.14475	509.9885
BF16H1	0.14116	513.6123
Mean	*0.18643*	*789.55*
STD	*0.07336*	*337.44*

E. Pull-Out Mechanical Setup



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WEIGHT:
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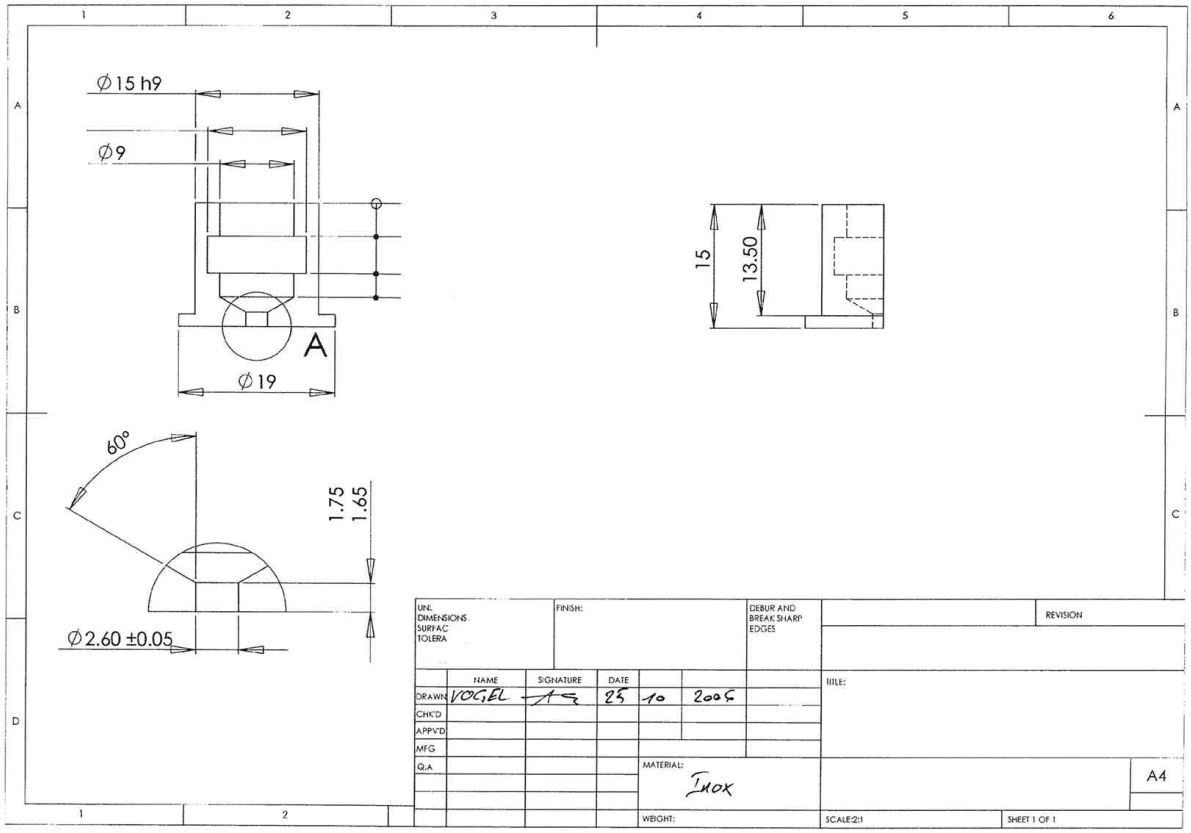
SHEET 1 OF 1


Ø15 h9
Ø9
A
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15
13.50
60°
1.75
1.65
Ø2.60 ±0.05
UNL DIMENSIONS SURFAC TOLERA
FINISH:
DEBUR AND BREAK SHARP EDGES
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NAME
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DRAWN VOGEL 25 10 2006
CHK'D
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Q.A
TITLE:
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A4
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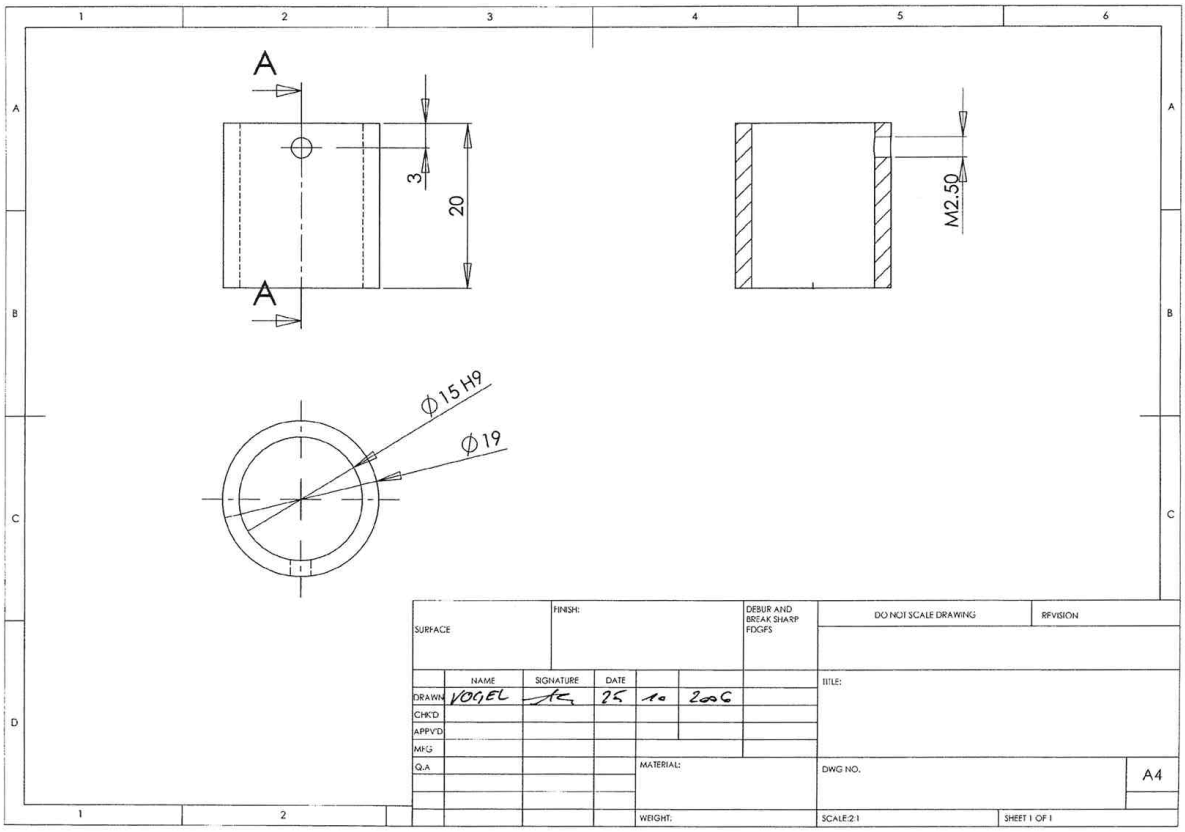
SHEET 1 OF 1


A
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SURFACE
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REVISION
NAME
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DRAWN VOGEL 25 10 2006
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A4
WEIGHT:
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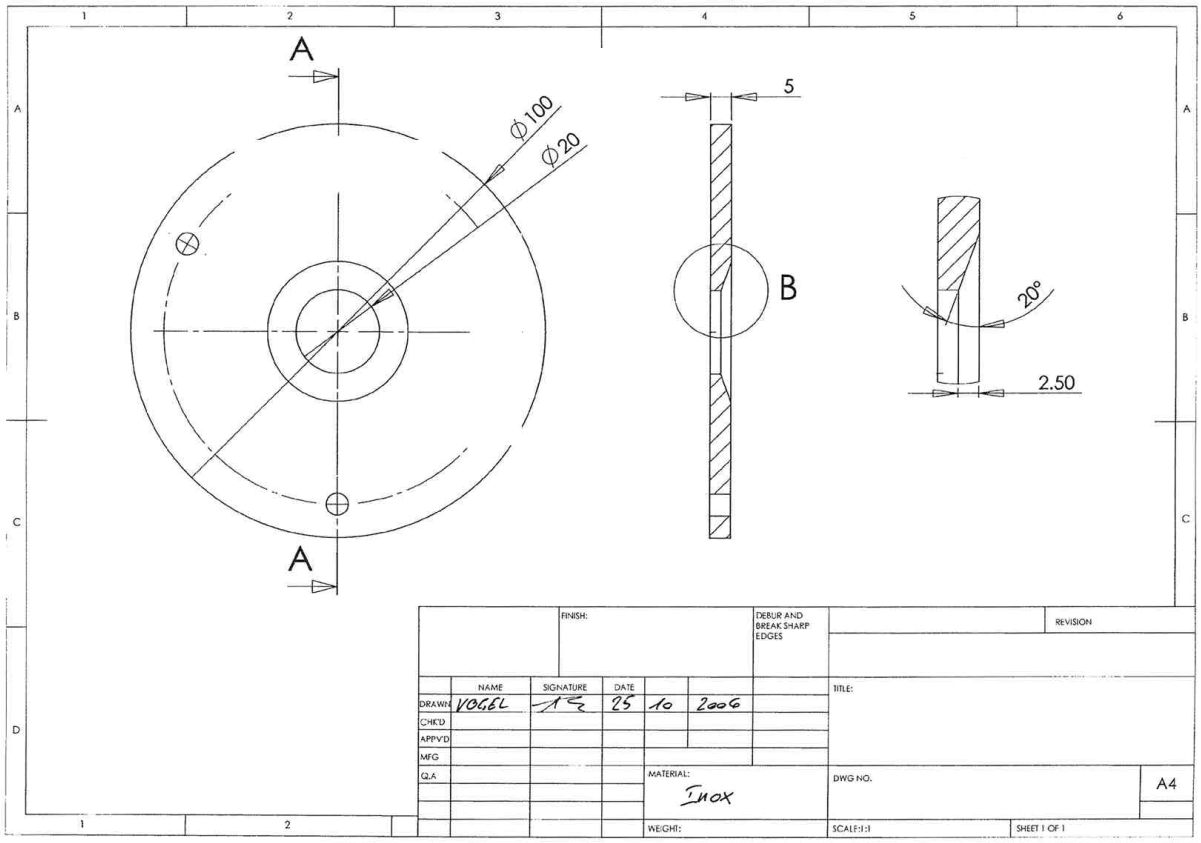
SHEET 1 OF 1



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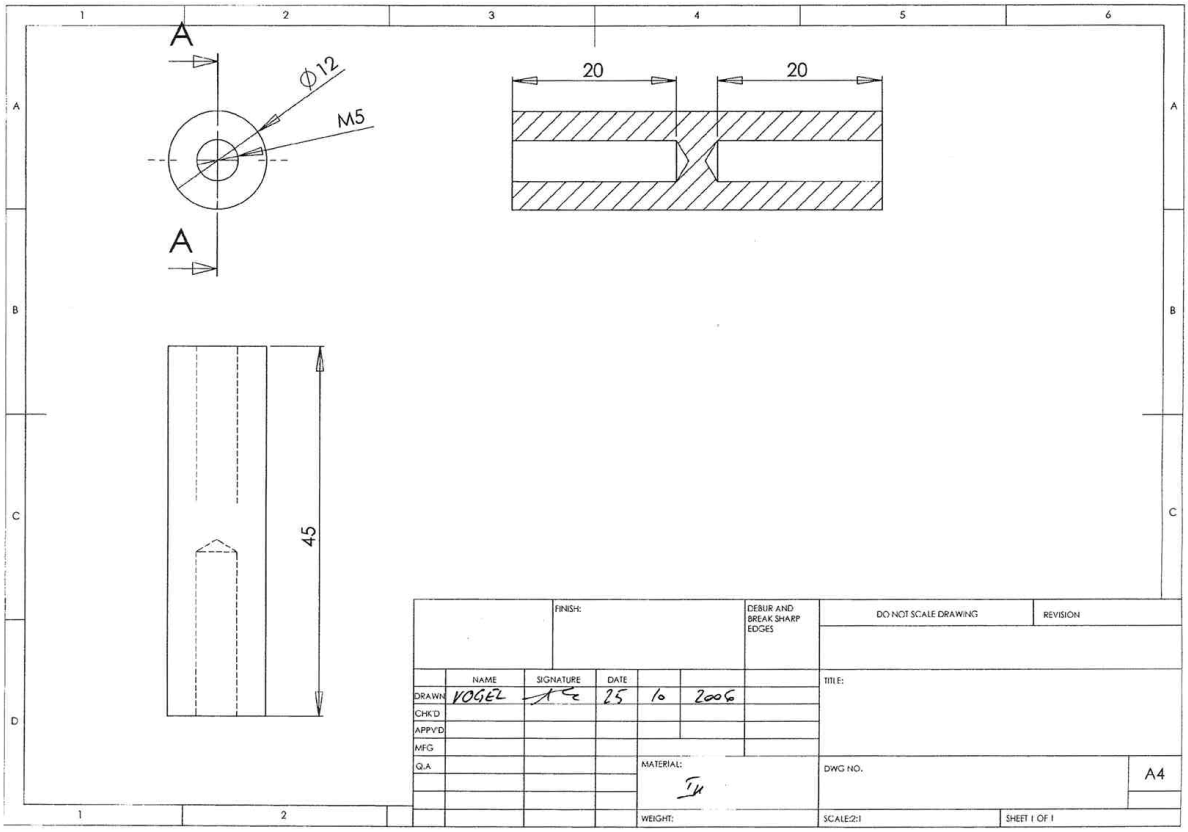

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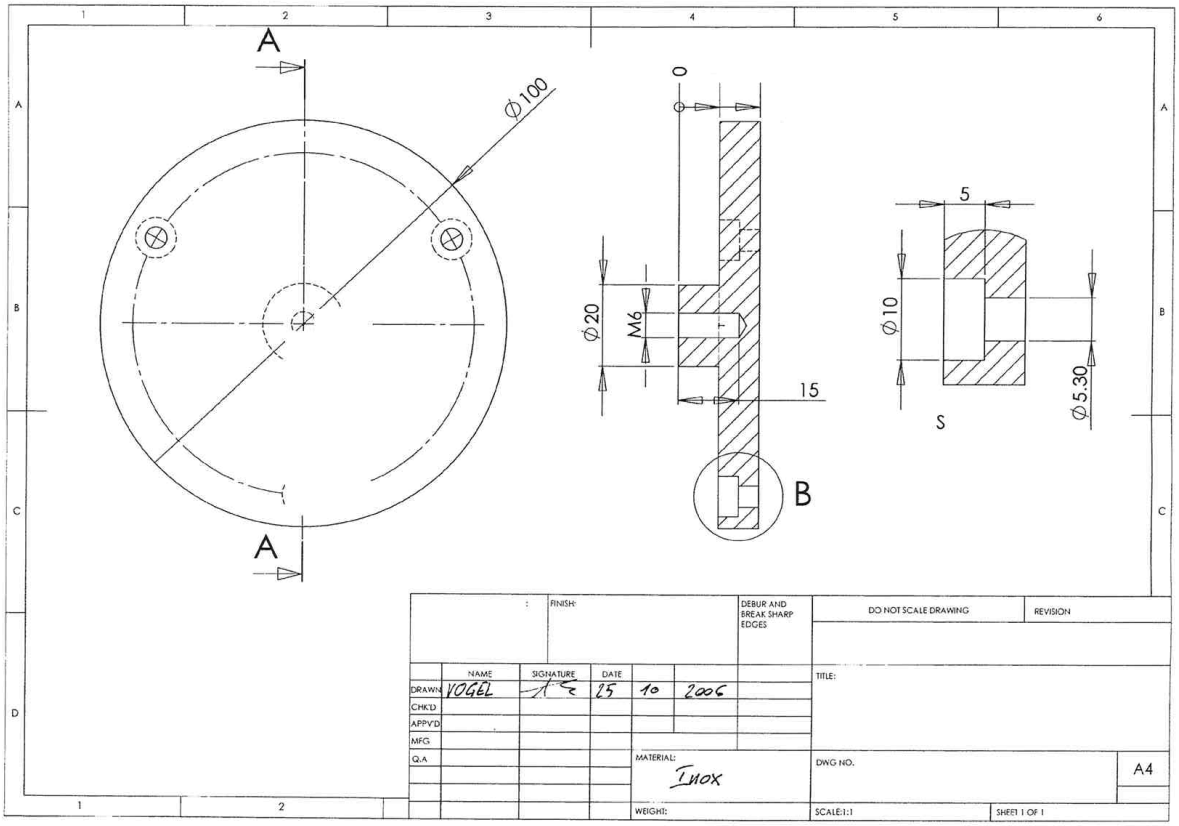

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CHK'D								
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MFG								
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