Influence of Calcium Phosphate on Osteogenesis in Hydrogel with Bone Morphogenetic Protein-2

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A large number of non-healing bone defects are due to injuries and diseases. These are generally treated by using autograft techniques, which is an invasive and painful treatment for the patient. It is important to evolve a new minimally invasive treatment for bone regeneration.

There are several studies investigating polymers as carriers for growth factors such as bone morphogenetic protein-2 and there are also plenty of studies about different calcium phosphates such as β-tricalcium phosphate and hydroxyapatite as implants. This study combined the two fields by examining the influence of different calcium phosphate additives into a hydrogel with a growth factor bone morphogenetic protein-2. I was interested in the influence of the different calcium phosphate compositions such as β-tricalciumphosphate and hydroxyapatite on osteogenesis. I also investigated the influence of the morphology of the hydroxyapatite on osteogenesis. I did this by comparing sizes and shapes of the hydroxyapatite. I also wanted to study what concentration of calcium phosphate was optimal for the bone formation. To answer these questions I injected bilaterally 0.2 ml of hydrogel with different concentrations and compositions of calcium phosphates into quadriceps muscle of rats. The animals were sacrificed after four weeks and examined with peripheral quantitative computer tomography. The results showed a significantly higher bone density for nano-sized hydroxyapatite, and samples with calcium phosphates resulted in greater bone formation with respect to volume, density and mineral content compared to hydrogel with bone morphogenetic protein-2 alone. There were also many indications that different calcium phosphate additives affected the bone formation differently, but I did not get any statistically significant results.

Summary

A large number of non-healing bone defects are due to injuries and diseases. These are generally treated by using autograft techniques, which is an invasive and painful treatment for the patient. It is important to evolve a new minimally invasive treatment for bone regeneration.

There are several studies investigating polymers as carriers for growth factors such as bone morphogenetic protein-2 and there are also plenty of studies about different calcium phosphates such as β-tricalcium phosphate and hydroxyapatite as implants. This study combined the two fields by examining the influence of different calcium phosphate additives into a hydrogel with a growth factor bone morphogenetic protein-2. I was interested in the influence of the different calcium phosphate compositions such as β-tricalciumphosphate and hydroxyapatite on osteogenesis. I also investigated the influence of the morphology of the hydroxyapatite on osteogenesis. I did this by comparing sizes and shapes of the hydroxyapatite. I also wanted to study what concentration of calcium phosphate was optimal for the bone formation. To answer these questions I injected bilaterally 0.2 ml of hydrogel with different concentrations and compositions of calcium phosphates into quadriceps muscle of rats. The animals were sacrificed after four weeks and examined with peripheral quantitative computer tomography. The results showed a significantly higher bone density for nano-sized hydroxyapatite, and samples with calcium phosphates resulted in greater bone formation with respect to volume, density and mineral content compared to hydrogel with bone morphogenetic protein-2 alone. There were also many indications that different calcium phosphate additives affected the bone formation differently, but I did not get any statistically significant results.
1 Introduction

1.1 Hydrogel as delivery system of bone morphogenetic growth factor 2.

The Division of Polymer Chemistry at the Uppsala University practises research in the development of polymeric materials that can be used in regenerative medicine. A biocompatible gel based on modified hyaluronic acid and polyvinyl alcohol (Bergman et al 2009) was developed through collaboration between researchers in the Polymer Chemistry group. The injectable hydrogel that they developed contains a modified hyaluronic acid with an aldehyde group (HAA) and polyvinyl alcohol with a hydrazide group (PVAH) (Bergman et al 2007, Ossipov et al 2007). Mixing aqueous solutions of the polymers causes gel formation in 1-2 minutes by cross-linking through hydrazone formation (Figure 1), which makes it ideal for injection. It has also been shown that the gel has good qualities to keep additives such as growth factors and ceramic particles, calcium phosphate (CaP), due to mechanical and electrostatic forces (Bergman et al 2007). Previous studies have shown a unique ability to induce bone formation in cranial defects in mini pigs that do not heal by themselves, called critical size defects (unpublished, Docherty, Engstrand, Bergman,) and ectopic bone formation in rats (Bergman et al 2009). This was achieved by adding the growth factor, bone morphogenetic protein-2 (BMP-2) (Urist 1965, Wozney, 1992) and calcium phosphate into the hydrogel. The calcium phosphate resembles natural bone mineral.

![Chemical reactions in formation of synthetic hydrogel. The cross-linking between the aldehyde-modified hyaluronic acid and hydrazide-modified polyvinyl alcohol. The red circles mark the modified groups and the hydrazone of the cross-linked polymer (reprinted with permission from Bergman et al. 2009).](image-url)
1.2 Calcium phosphates

CaP₃s are known for their conductivity of osteogenesis (LeGeros 2008). These studies have provided indications that calcium phosphate has a major impact on the density and growth rate of the newly formed bone. There are a number of bio-ceramic materials such as betartricalciumphosphate (β-TCP), Ca₃(PO₄)₂, (Metsger et al. 1982), which is known for its osteoconductivity as a ceramic implant through its high solubility. This makes β-TCP more biodegradable through the cellular activity and the acidic environment. This causes the β-TCP to disassociate into calcium and phosphate ions that saturate the environment. These ions assemble and precipitate to form biological apatite also known as carbonate hydroxyapatite (CHA), which has the approximate formula (Ca, Na Mg)₁₀(PO₄, HPO₄, CO₃)₆(OH,Cl, F)₂, (LeGeros. 2008). Only CHA is present in healthy calcified tissue. Hydroxyapatite (HAP) Ca₁₀(PO₄)₆(OH)₂ (Kay et al. 1964) is another CaP₃ that is more stable and thereby less biodegradable than β-TCP. The structure of HAP is similar to that of the endogenous carbonate hydroxyapatite (LeGeros 2008). Biomimetic HAP has a morphology similar to the nano needles of biological apatites in bone. Ostim® consists of a paste containing 65 % sterile water and 35 % biomimetic hydroxyapatite (BHAP) in a syringe. The different characteristics of the CaP₃ make it important to understand what properties are critical for bone induction and bone conduction (material that convey the bone tissue). At this point the treatment for non-healing injuries is autograft where you often take grafts from the pelvic bone. This an invasive and aggressive method that often keeps the patient confined to bed for a long time. Consequently there is a need of further studies for the development of the optimal materials for repair of recalcitrant and complex bone injuries.

1.3 Peripheral quantitative computer tomography

To evaluate bone tissue a type of X-ray computer tomography (CT) called peripheral quantitative computer tomography (pQCT) is used. It operates in the same way as a regular CT by scanning a section of the body or sample in 360 ° angel. The CT sends X-ray beams through the sample and then registers the attenuation of the beams after passing through. This gives data in slices where you can see for example the bone mineral density (BMD) of each slice by calculating the attenuation of the beam correlated to the density of the tissue. This is a complicated calculation that the machine provides. The slices are often 1 mm sections of the whole sample (Appendix Fig A1). You can also analyse bone mineral content and area of the bone of each slice and then calculate the bone volume by multiplying the area by the width of the slice.

Aims

The aim of the study was to answer the following questions: Will different CaP₃ additives affect bone density and structure? Which CaP₃ properties are critical for bone formation (size, area or chemical composition)? What is the optimal concentration of hydroxyapatite?

The study attempted to answer these questions by comparing the ability to form ectopic bone in different groups of calcium phosphate combinations in rats.
2. Results

2.1 Scanning Electron Microscopy images of dried Ostim®

Before I could prepare the hydrogels with additives I dried the commercial Ostim® preparation, which consists of biomimetic hydroxyapatite (BHAP) needles dispersed in sterile water. Scanning electron microscopy (SEM) was used to investigate the properties of the dried Ostim® that was to be used to stimulate ectopic bone production and the untreated Ostim® before drying. The SEM images showed rod-like hydroxyapatite mono-needles in the untreated Ostim® paste (fig. 3), which were similar in morphology to the carbonate hydroxyapatite needles of the bone. After drying the Ostim® paste, the morphology changed as the BHAP needles were aggregated into flakes, but the biomimetic needle shape of the hydroxyapatite appeared to be intact (figure 4). The morphology seemed promising for the forthcoming biological studies.

![Fig 3](image3.png)

Fig 3 Scanning electron microscopy image of Ostim® paste. The magnification was 55000 X.

![Fig 4](image4.png)

Fig 4 Scanning electron microscopy image of dried and pulverized Ostim® (BHAP). The magnification was 55000 X.
2.2 Effects of different hydrogel additives on ectopic bone formation

I then investigated the importance of various crystal phases of HAP and β-TCP and their particle sizes (micrometer or nanometer in diameter). I choose clods of HAP greater then 100 μm (provided by Olof Eriksson Department of Material Sciences, Uppsala University) and nano-sized HAP with a size of 20 nm in diameter NanoHAP (provided by Qinghong Hu, Department of Chemistry, Zhejiang University, China). I also investigated the importance of mimicking nature by using biomimetic hydroxyapatite needles (BHAP). The optimal concentration for bone formation of hydroxyapatite in the hydrogel was explored with three different concentrations of HAP, amounting to five, ten and 25 weight-percent of the hydrogel. Material was injected into the femoral muscles and individuals were sacrificed after four weeks (fig. 5). Tissue samples were analyzed in terms of density, mineral content and bone volume using peripheral quantitative computer tomography at Akademiska Sjukhuset Uppsala University.

![Fig. 5 Polymers in dual compartment syringes injected bilaterally into quadriceps muscles of rat for cross-linking in situ. (Image reprinted with permission from K Bergman 2010).](image)

The dissected legs were placed on an X-ray plate and X-rayed to localize where the ectopic bone had been developed. There was a distinct white shadow on the X-ray pictures of newly formed bone (figure 5). The shapes of the newly formed bone were polymorphic and they were located at different sites in the quadriceps. This was most likely due to the injection
technique that made it difficult to inject at the exact same location on the quadriceps. This problem made it difficult to quantify the ectopic bone.

![Image of hydroxyapatite additves in hydrogel injected into quadriceps muscles of rat.](image)

Fig. 5 Hydroxyapatite additives in hydrogel was injected bilaterally into quadriceps muscles of rat. The animals were sacrificed four weeks later and their legs subjected to X-ray analysis. The newly formed bone appears as an white shadow next to the femoral bone the picture to the left is legs from various groups to illustrate the diverse morphology and location. Picture to the right is a close up of a quadriceps muscle with hydrogel and β-TCP as a CaP, additive.

The X-rayed legs was further analysed by pQCT and the results was analysed and evaluated statistically. There was a wide distribution of the values in the three parameters density, volume and mineral content in all the groups (fig. 6-11). The 5 % hydroxyapatite group was excluded from the results due to misleading data because of poor injection technique.

### 2.3 Bone density.

The nanoHAP yeilded a significant difference in bone density compared to all the groups except for the Ostim® group (figure 6). ANOVA Tukey's multiple comparison test gave a p-value of 0.0025 when comparing the groups of nanoHAP with 25 %HAP, β-TCP and Clods. There was a significantly higher level of bone density with nanoHAP than with 25 % HAP (p<0.01). When comparing nanoHAP to β-TCP and clods the p-value was below 0.05 (fig. 6). Unpaired t-test showed a significant difference between nanoHAP and control (containing no HAP; p= 0.01; fig. 7). There were no statistically significant results between the different concentrations only indications that a higher volume of hydroxyapatite obtained greater bone formation.
The lowest values of bone formation (bone density, mineral content and bone volume) of the samples were lower than the amount of injected CaP. This indicated that these low mineral content values were due to failed injection, where the hydrogel got injected outside of the analysed quadriceps muscle, probably subcutaneously or other locations in the leg. If the sample were injected correctly the mineral content of the additive itself would have been detected as a mineral content in the pQCT. To avoid misleading data due to failed injections a lower third was excluded in all the groups to treat the groups equally. When the lower third of the values were excluded a significant difference was observed using ANOVA Tukey’s multiple comparison test between the 25% HAP and the Ostim® group (p< 0.01). Also the difference between the Ostim® group and β-TCP was significant (fig. 8).

Fig. 6. Density of ectopic bone formed upon injection of rat quadriceps muscle with hydrogel additives. Injection and sample preparation were as described in the legend to fig 5. All additives were tested at 25 % concentration: hydroxyapatite (HAP), clods of hydroxyapatite greater than 100 μm (Clods), nano hydroxyapatite with diameter of 20 nm (nanoHAP), b-tricalciumphosphate (β-TCP) and biomimetic mono-needles of hydroxyapatite (Ostim). The samples were scanned with pQCT and the results were statistically analysed by ANOVA Tukey’s Multiple Comparison Test. The arrows with stars represents significance level between the groups from which the arrow begins to the group where the arrow points; *=p <0.05; **= p <0.01.
2.4 Bone mineral content.

The groups with the different hydrogel additives included samples with low mineral content, which gave a wide distribution in all the groups with hydrogel and additives. The mineral content was measured in milligrams per millimetre (of the 1 mm thick slices which were analysed). The variation within the groups was high and there were only indications that the different additives affected the mineral content of the bone. The lowest third of the values
were excluded due to low mineral content. A significantly higher mineral content was observed in the 25% HAP group compared with nanoHAP using a unpaired t-test (p=0.03, fig. 9).

![Bone Mineral Content](image)

Fig. 9 Bone mineral content of the same samples with different hydrogel additives analyzed in fig 6. Injection and sample preparation were as described in the legend to fig 5. The samples were scanned by pQCT and the lower third of values were excluded. The results were analysed by unpaired t test. The arrow with stars represents significance level between the groups from which the arrow begins to the group where the arrow points, *=p-value <0.05.

### 2.5 Bone volume

The bone volume was measured in mm³. There were many indications that the different additives had effect on the volume of the newly formed bone. There was a wide variation within the individual groups resulting in a non-significant outcome. The values were scattered except for the nanoHAP group where the values were clustered. One third of the lowest values were excluded due to the low mineral content. A significant difference could be observed between 25 % HAP and control and between 25 % HAP and nanoHAP. Both were evaluated in an unpaired t test which gave a significant difference between control and 25 % HAP (p=0.04, fig. 10). There were also a difference between 25 % HAP and nanoHAP, (p=0.0144, fig. 11).
Fig. 10 Bone volume of the same samples with different hydrogel additives analyzed in fig 6. Injection and sample preparation were as described in the legend to fig 5. The samples were scanned by pQCT and the lower third of values were excluded. The results were analysed by unpaired t test. The arrow with stars represents significance level between the groups from which the arrow begins to the group where the arrow points, *=p-value <0.05.

Fig. 11 Bone volume of the same samples with different hydrogel additives analyzed in fig 6. Injection and sample preparation were as described in the legend to fig 5. The samples were scanned by pQCT and the lower third of values were excluded. The results were analysed by unpaired t test. The arrow with stars represents significance level between the groups from which the arrow begins to the group where the arrow points, *=p-value <0.05.
3. Discussion

Having verified that dried Ostim® formed biomimetic flakes it was interesting to observe that it produced bone with greater density compared to 25% HAP and β-TCP. There was no significant difference compared to nanoHAP, only indications that nanoHAP had a higher bone density. We can conclude from this data that biomimetic HAP (Ostim®) is favourable for osteogenesis instead of the pure HAP or β-TCP. Earlier studies with hydroxyapatite coatings has demonstrated that there was a high ingrowth of bone when biomimetic hydroxyapatite was used (Huber et al, 2009, Hermida et al, 2005).

Addition of CaPs to the hydrogel resulted in greater bone density. There were also indications that certain CaPs has a greater impact on bone density then others. The nanoHAP group stood out with a higher bone density then the rest of the groups implying that osteogenesis proceeds better with nano-sized HAP. This shows that nanoHap and the Ostim® are the best additives regarding bone density. There is also indications that the properties of nanoHAP is favourable for osteogenesis compared to Ostim® and that the size of the hydroxyapatite particles has a major impact on the bone density. The biomimetic properties of Ostim® may lead to an advantage by higher bone ingrowth due to a greater osteoconductivity and that could be an important factor for the bone density. The properties of nanoHAP could be a chemical reaction where the nanoHAP by its small particle size gives a very large area compared to the other groups. This could give a chemical effect in the surrounding fluids of the extracellular matrix where the nanoHAP will bind calcium and phosphate ions to the surface of the NanoHAP particles, and this will cause a decrease. It has been reported that a small decrease of calcium and phosphate ions will signal to mesenchymal stemcells to differentiate into osteoblast (Yukan et al, 2008).

The major conclusion from this study is that CaPs in hydrogel definitely have a positive influence on the bone formation even though the BMP-2 itself manages to provide new living bone.
4. Materials and methods

4.1 Biological material

The study involved eight groups with three animals in each group (table 1). Animal procedures were approved by Uppsala ethical committee (249/8). The Male Sprague Dawley rats weighing 400-450 g were obtained from NOVA-SCB AB. The animals were kept by three in macron 4 cages at the animal facility at Rudbeck laboratory.

Table 1. Additives and their use

<table>
<thead>
<tr>
<th>Additive</th>
<th>Abbreviation</th>
<th>Weight (%)</th>
<th>Animals</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No HAP</td>
<td>0</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Hydroxyapatite 5 %</td>
<td>HAP 5%</td>
<td>5</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Hydroxyapatite 10 %</td>
<td>HAP 10%</td>
<td>10</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Hydroxyapatite 25 %</td>
<td>HAP 25%</td>
<td>25</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Nano hydroxyapatite</td>
<td>NanoHAP</td>
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<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Hydroxyapatite clods &gt; 100 µm</td>
<td>Clods</td>
<td>25</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Beta-tricalciumphosphate</td>
<td>Beta-TCP</td>
<td>25</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Ostim® biomimetic hydroxyapatite</td>
<td>Ostim®</td>
<td>25</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

4.2 Preparation of Ostim®

1.2 g of Ostim® paste (Aap Implantate AG) was dried for 2 days in an oven at 180 °C in 3 ml glass tube (Vial, Wheaton US) sealed with aluminium foil to keep it sterile. The dried Ostim® was pulverized in a mortar. Scanning electron microscope (SEM) images were prepared at a magnification of 55k, an EHT voltage of 3 kV and a working distance of 4 mm.

4.3 Sterilisation of calcium phosphate additives.

The hydroxyapatite (Amorphous Calcium Phosphate, Plasma-Biotal Ltd) and each additive were measured (see table 2 for additives and providers) and transferred to 3 ml glass tubes and sealed with aluminium foil. All the glass tubes containing the additives were then transferred to a 500 ml glass container (Duran®, Germany), sealed with aluminium foil and placed in an oven at 180 °C for 3 days for sterilisation.
Table 2. Amounts of additives.

<table>
<thead>
<tr>
<th>Additive</th>
<th>Provider/ Provided by</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyapatite, 5 %</td>
<td>Amorphous Calcium Phosphate, Plasma-Bial Ltd</td>
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<tr>
<td>Hydroxyapatite, 10 %</td>
<td>Amorphous Calcium Phosphate, Plasma-Bial Ltd</td>
<td>0.301</td>
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<tr>
<td>Hydroxyapatite, 25 %</td>
<td>Amorphous Calcium Phosphate, Plasma-Bial Ltd</td>
<td>0.750</td>
</tr>
<tr>
<td>Nano Hydroxyapatite, 25 %</td>
<td>Qinghong Hu, Department of Chemistry, Zhejiang University, China</td>
<td>0.750</td>
</tr>
<tr>
<td>Hydroxyapatite Clods &gt;100 µm, 25 %</td>
<td>Olof Eriksson Department of Material Sciences, Uppsala University</td>
<td>0.751</td>
</tr>
<tr>
<td>β-TCP, 25 %</td>
<td>&quot; Olof Eriksson Department of Material Sciences, Uppsala University</td>
<td>0.750</td>
</tr>
<tr>
<td>Ostim® (BHAP), 25 %</td>
<td>Aap Implantate AG</td>
<td>0.750</td>
</tr>
</tbody>
</table>

4.4 Preparation of gels

For each injection 0.5 ml of hydrogel was required. For the trial a total amount of 30 ml hydrogel was used with 150 µg/ml bone morphogenetic protein-2 (BMP-2) (Inductos, Wyeth). 49.5 mg polyvinyl alcohol (PVAH) (provided by Kristoffer Bergman, division of polymer chemistry, Uppsala University) was dissolved to a concentration of 3.3 mg/ml in 15 ml BMP-2 formulation buffer (InducOS™, Wyeth Europe Ltd.), 400.5 mg aldehyde modified hyaluronic acid (HAA) (provided by Kristoffer Bergman, division of polymer chemistry, Uppsala University) was dissolved to 26.7 mg/ml in 15 ml PBS (0.01 M phosphate buffered saline (NaCl 0.138 M; KCl 0.0027 M; pH 7.4)). The components were filtered sterilized through a 0.45 µm syringe filter (Whatman® Puradisc™ FP30 syringe filters). Both components were transferred to 15 ml Falcon™ tubes (Falcon™, BD Biosciences) where the CaP₅ were added and vortexed (MS1 minishaker IKA®, Tamro Medlab Sweden). The components were transferred to 1.75 ml MiniMix™ dual compartment syringes (TAH Industries Inc.). 0.25 ml of each hydrogel component was transferred with a 1 ml syringe (Luer BD Plastipak™, Spain) and a 18 G needle (Noelus®, Terumo Europe N.V.) to the dual syringe to a total volume of 0.5 ml hydrogel to take account of the dead volume of 0.3 ml in the mixing tip when injected. Everything was performed under aseptic conditions. The hydrogel syringes were left overnight at 4°C to be injected the day after.
4.5 Injections and animal study.

Animals were weighed and then anaesthetised with isofluran (Forene®, Abbot Scandinavia Sweden) beginning with 4 l/min oxygen and 4 l/min isofluran in an induction chamber and then using a mask with 1.5 l/min oxygen, 1.5 l/min air and 3 l/min isofluran. To confirm surgical anaesthesia the rats were pinched in between their toes. Lack of withdrawal reaction indicated anaesthesia. Each rat was placed supine and the thighs were shaved and washed with 70% ethanol. The dual compartment syringe was placed in a syringes holder. A mixing tip and a 21 G needle (Noelus® © Terumo Europe N.V.) were placed on the dual compartment syringe. To find the right location for injection, the quadriceps was fixed and the needle was inserted through the muscle to the femur and then withdrawn back into the muscle. The hydrogel was then injected into the muscle. The rats were injected bilaterally. After injection the rats were analgised with buprenorfin 0.05 mg/kg (Temgesic®, injection agent solution 0.3 mg/ml. Shering-Plough Europe) subcutaneously. The animals were allowed to move freely in Macron 4 cages after injection and were monitored daily by animal facility staff. The animals were sacrificed after 4 weeks in a CO₂ chamber. The legs were dissected at the condyl and femoral head to collect only the femur and quadriceps muscle. The legs were preserved in 4% paraformaldehyde (Histolabs product AB).

4.6 X-ray analysis

The legs were mounted in 50 ml falcon tubes (Falcon™, BD Biosciences) using pieces of Styrofoam. The legs were X-rayed at tube voltage 40 kV for 1 sec with tube current 3 mA in an X-ray machine (Cabinet X-ray system Faxitron Series, Hewlett Packard US) to show the position of the newly formed bone in the muscle. X-ray plates with femur and quadriceps muscle were used to locate newly formed bone.

4.7 Peripheral quantitative computer tomography

The legs were scanned by peripheral quantitative computer tomography (pQCT) (XCT Research SA+ Stratec, Germany) at the known position taken from the X-rays. This was done first to get an overview of the bone, called scoutview, to obtain the location of the bone and thereby know where to analyse. The pQCT was set to cover the whole bone formation (fig 5). voxel size (computer analysing unit) was 0.10, the space between slices was 1 mm and the number of slices was between 15 and 27 due to the shape of the ectopic bone. For more detailed pQCT setting see Appendix (Fig A1)

4.8 Data processing

Data were processed using a program for the pQCT, XCT Stratec Console software (Stratec, Germany), with a threshold of 280, peel mode 2, TreshCRT 240 and cortmode 2. The data was saved to an Excel file where the data were tabulated and interpreted into bone density (mg/cm³), bone mineral content (mg/mm) and bone volume (mm³).

4.9 Statistical evaluation

The results were evaluated by the statistical software Prism 5.0 (Graph Pad; Software inc USA). The statistical method chosen was a non-parametric one-way ANOVA, the Kruskal
Wallis test with non Gaussian approximation and Dunns post test with significance level 0.05 (95% confidence intervals). I also used an unpaired t-test with significance level of 0.05 to see if there was any significant variation between groups.
5 Acknowledgments

Thanks to….
To my wonderful boyfriend Wictor and my beautiful son Viggo, the two persons I love the most. To my wonderful family which have supported me during my whole life.
To my eminent supervisor Kristoffer Bergman, who put up with me and my amazing former chief Jöns Hilborn for all the inspiration, help and enthusiasm. To all my former colleagues at the Division of Polymer Chemistry and my new colleagues at Akademiska Sjukhuset.
Especially thanks to Britt-Marie for all the help with the pQCT. BIG THANKS to Sune Larsson and Kenneth Jonsson who have supported me and pushed me to finish my studies.
HUGS to you all!
6 References


Fig. A1. One slice from the sample is shown in the left of the picture. Two white circles represents a section of the femur and the other a section of the newly formed ectopic bone. On the right is shown a whole bone in an overview called scout view. The scout view is used to localize where to analyse the sample.
Fig. A2. Peripheral quantitative computer tomography settings. This figure is a screen save from the program operating the pQCT. Here it is possible to set where on the sample the pQCT should start to screen and what kind of information you are looking for.