DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
140

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140

HETEROTOPIC OSSIFICATION AFTER TOTAL HIP ARTHROPLASTY: CLINICAL AND PATHOGENETIC INVESTIGATION

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CONTENTS

To my progenitors and differons

LIST OF ORIGINAL PUBLICATIONS

I Toom A, Haviko T, Rips L. **Heterotopic ossification after total hip arthroplasty.** *Int Orthop*, 2001; 24(6) 323–6

II Toom A **Heterotoopse luustumise hindamine: erinevate klassifikatsioonide võrdlus [Assessment of heterotopic ossification: comparison of different classifications***; article in Estonian***].** *Eesti Arst*, 2003; Lisa 6: 7–11

III Toom A, Fischer K, Märtson A, Rips L, Haviko T. **Interobserver reliability in the assessment of heterotopic ossification: proposal of a combined classification.** *Int Orthop,* 2005;29(3):156–9

IV Toom A, Möls M, Fischer K, Uibo R, Veske K, Selstam G, Haviko T, Arend A, Märtson A. **Digital planimetry for determination the severity of heterotopic ossification and sources of assessment errors** (manuscript).

V Toom A, Arend A, Gunnarsson D, Ulfsparre R, Suutre S, Haviko T, Selstam G. **Bone formation zones in heterotopic ossifications: histologic findings and increased expression of BMP-2, TGF-β_2 and TGF- β_3.** *Calcif Tissue Int,* 2007; 80(4):259–67

VI Toom A, Suutre S, Märtson A, Haviko T, Selstam G, Arend A. **Osteoprogenitor cells for the heterotopic ossification in the experimental rat model** (Submitted to *Acta Orthopaedica*).

ABBREVIATIONS

^{32}P-ATP	–	^{32}P-labelled adenosine triphosphate
ALP	–	alkaline phosphatase
BMP	–	bone morphogenetic protein
BV/TV	–	ratio of bone volume to total sample volume
CI	–	confidence interval
COX-1/2	–	cyclooxygenase-1/2
CT	–	computerized tomography
EDTA	–	ethylenediaminetetraacetic acid
HO	–	heterotopic ossification
IL-1	–	interleukin-1
mRNA	–	messenger ribonucleinic acid
Md.V/TV	–	mineralized volume ratio to total sample volume
NSAID	–	non-steroidal anti-inflammatory drugs
rhBMP	–	recombinant human bone morphogenetic protein
OS/Es	–	ratio of osteoid surface to endostal surface
OS/Ps	–	ratio of osteoid surface to periostal surface
Ob.S/BS	–	ratio of osteoblast surface to bone surface
OS/BS	–	ratio of osteoid surface to bone surface
OV/BV	–	ratio of osteoid volume to bone volume
RNA	–	ribonucleinic acid
RT-PCR	–	reverse-transcription polymerase chain reaction
TGFβ	–	transforming growth factor beta
THA	–	total hip arthroplasty

INTRODUCTION

Heterotopic ossification (HO) refers to formation of bone in tissues, which normally are not ossified. HO was first mentioned in the literature as *myositis ossificans progressiva* syndrome in the year 1692 by Patin [Geschickter & Maseritz 1938]. Riedel later provided a more detailed description of HO after neurologic injury [Riedel 1883]. HO can occur in many tissues and Binnie thoroughly described the appearance of HO after traumas (Binnie 1903). Déjerine and Ceiller were the first to describe heterotopic ossification in paraplegic patients [Déjerine & Ceiller 1919].

HO as a complication of total hip replacement was mentioned for the first time in 1951 [McKee 1951]. From a historical point of view it should be mentioned, that the first suggestion for prophylaxis was made in 1961, when Damanski reported that a more adequate treatment of traumatic injuries might decrease the incidence of HO [Damanski 1961].

Practising doctors and researchers often face difficulties in precise differentiation between the heterotopic calcification and ossification process. Many factors contribute to the heterotopic bone formation and the process of HO is so much heterogenous, that it is even difficult to define it properly. Therefore the definition of Benjamin Shaffer is often preferred, stating: "Heterotopic ossification refers to the formation of bone in tissue, which is usually unossified" [Shaffer 1989]. However, during the last decades our ability to investigate bony structures, their function and management has improved substantially.

Firstly, efforts in material technology, improvement in imaging techniques and continuously promoted educational and communicational activities have helped surgeons to perform operations in situations where earlier only conservative options were available. Also, the goals of surgery are more concise and therefore demand evidence-based information. Secondly, novel technologies are continuously introduced in bone research. New tissue preparation methods allow us to more delicately intrude into the *nature mort* of bone cells. Imaging techniques help us to have insight into micro- and nanostructures. Thus, the developments look promising. Despite the fact that the HO syndrome has been known for a long time our understanding of the entity of HO and its pathogenesis is limited as the formation of HO contains complicated interactions both within ossificates and with the surrounding tissues.

This thesis focuses on practical questions that arose during the management of the heterotopic bone formation, as there were no previous investigations performed in this field in our clinic and treatment strategies reported in the literature were disputable. Also, the question of HO assessment was purely

practice related, as we experienced the difficulties related to insufficient reproducibility of our HO diagnoses.

When investigating this heterogenous process we tried to find out some common entities of the HO induction and formation as well basic triggers of the process. It is truly hoped that the findings of this study can add some “piece” to our progressively growing “knowledge-mosaic” of heterotopic ossification.

REVIEW OF LITERATURE

1. Heterotopic ossification – what is it?

HO is defined as the formation of bone inside soft-tissue structures where it normally does not occur. It refers to formation of lamellar bone in the extra-skeletal tissues. The main criteria for HO morphologic diagnosis are presence of bone cells and collagenous matrix as well as formation of hydroxyapatite crystals, but the absence of cellular and even tissue atypism [Bosse 1997, Puzas *et al* 1989].

Ectopic calcification is mineralization of soft-tissue structures, which usually follows chemical or physical trauma, as in tendinitis calcarea. Histologically, a calcium deposit rather than new bone will be formed [Vanden Bossche & Vanderstraeten 2005]. These two conditions, HO and ectopic calcification, should be clearly differentiated.

Usually HO is appearing in the tissues of mesodermal origin like muscle or connective tissue [Petty 1991].

In clinical practice there are three main causative factors: traumatic, genetic and neurogenic [Balboni *et al* 2006].

HO can be classified on the basis of evoking agents and by the extent of its spreading [Bosse 1997, Puzas *et al* 1989, Thomas 1992]:

a) generalised HO caused by systemic illnesses and conditions (paresis and paresthesia caused by injuries of central nervous system, *fibrodysplasia ossificans progressiva* etc.)
b) local HO as a result of a local trauma (fractures, surgical trauma like THA, muscle distorsion, burns etc.)
c) local HO as a result of a local metabolic or organic changes in tissues (e.g. continuous overuse or micro-traumas, intramuscular and subcutaneous injections, tumors etc).

Pathogenesis of posttraumatic, postoperative, neurologic and idiopathic HO is somewhat different but there are, however, also several common factors for these conditions.

The current dissertation first of all concerns the HO occurring after total hip arthroplasty (THA), i.e. the type of HO belonging to the group of local HO caused by a surgical trauma.

The clinical diagnosis of HO is based first of all on the x-ray findings. However, on x-ray HO can be detected only 3–6 months after its induction. That corresponds to the term when at least some parts of HO have been completely formed [Adler 2000]. It has been demonstrated in patients with cerebral infarction that bone scans can reveal the forming HO already 3 weeks after occurrence of infarction [Orzel & Rudd 1985]. However, considering the

local tissue trauma occurring after THA the bone scans may be too unspecific for diagnosis of HO.

2. Classifications of HO

The most widely used classification was proposed by Brooker and co-authors in 1973 and is, despite its insufficiencies, still leading system used for that purpose.

The most common methods for quantitative and qualitative assessment of HO in clinical practice are based on comparing the frontal or plain x-rays of the proximal hip region made during follow-up and during postoperative period [Arcq 1973, Brooker *et al* 1973, DeLee *et al* 1976]. Currently, the most widely used classification for assessment of the HO is Brooker's system [Brooker *et al* 1973]. It was recently critically analyzed by Della Valle and co-authors [2002] and they proposed a simplified rating system. They reported a better differentiation between small ossifications, which hardly cause any clinical symptoms at all, from clinically more severe ossifications [Della Valle *et al* 2002]. The classification by Arcq, which is widely used in German-speaking countries [Arcq 1973] and a classification system published by DeLee and co-authors, which takes both severity and localization of HO [1976] into account, were also included in this study.

Classifications of HO. Brooker's classification divides HO into four classes: class I: islands of bone within soft tissues of any size; class II: bone spurs from pelvis or femur, leaving at least 1 cm between opposite bone surfaces; class III: the same as previous but reducing the space between opposite bone surfaces to less than 1 cm; and class IV: ankylosis [Brooker *et al* 1973].

Della Valle's classification divides patients into three groups: A: HO is absent, or there are present new bone islands less than 1 cm in diameter; B: isolated ossifications with a diameter at least 1 cm and marginally localized ossifications leaving at least an 1 cm distance between pelvis and femur; and C: marginally localized ossifications leaving less than 1 cm distance between pelvis and femur or to ankylosis [Della Valle *et al* 2002].

Arcq's classification does not specify the minimum size of ossifications. Class I refers to isolated or marginal ossifications, which "bridge" the opposite bone surfaces. Class II refers to the bony bridging in one side of the implant and class III on both sides of the implant [Arcq 1973].

DeLee's classification is more complicated: Class I: isolated ossifications in the soft tissues exceeding 5 mm; classes II and III: presence of ossifications on the lateral or medial side of the implant, respectively. Additionally, letters A and B, which refer to the localization of the ossification on the pelvis or femur, respectively, are added.

Arabic numberals added mark the sub-class of severity where "1" refers to extension of HO less than 50% of the pelvic-femoral distance, "2" refers to more than 50% and "3" refers to complete bridging [De Lee *et al* 1976].

In order to achieve a higher agreement in evaluation of morphological parameters in pathology, reliability should be established. The possibility to reproduce estimations is called reliability [Svanholm *et al* 1989]. For the estimation of reliability the kappa value is used. Cohen's kappa-coefficient has been applied to improve the criteria for development of classification systems [Cohen 1960, Svanholm *et al* 1989]. In the estimation of HO after total hip arthroplasty by the mostly used Brooker's classification demonstrated low reliability between repeated observations and 5s [Neal *et al* 2000b, Wright *et al* 1994]. In a large multicentre study the Cohen's kappa value has been calculated to be 0.50 (95% CI 0.30–0.70), which means „satisfactory" [Neal *et al* 2000b]. Brooker's classification was revised in 1994, by adding some clarifying criteria, but inter-observer reliability as measured by weighted kappa was increased from initial 0.57 only up to 0.68 [Wright *et al* 1994].

A large multi-centre study reports the interobserver reliability based on Cohen's kappa-value to be "fair" 0.50 (95%CI 0.30–0.70) [Neal *et al* 2000a].

The high variability generates the necessity to analyse what are the sources of errors. For adequate control of roentgenological estimations it is important to compare histological and histomorphometrical parameters to the CT findings. This material is available only in the hospitals where reoperations are performed. As such operations are seldom used in patients with un-complicated ossifications this methodology is not feasible.

For the classification of HO the quantitative method of Schoellner has been used, which is based on digitalized planimetry analysis [Schoellner *et al* 2000]. With this method ossifications can be divided into ten groups and classification is not considering the localization of HO. The minimal size of ossification that is possible to detect by the use of digitalized planimetry is as little as 0.1 cm^2 [Schoellner *et al* 2000].

The method is technically complicated and for this reason it is today not suitable for everyday use. The technique is also time-consuming. Digital planimetric estimation consists methodologically of two stages – diagnostics of image and technical subscription of marking the image. Results are given mathematically as estimated nominal values. Principally the same method was used in this dissertation to estimate a source of errors in classification of HO, which in detail is described in the chapter "Material and methods".

3. Incidence of HO

From the clinical point of view HO is a very commonly seen phenomenon after THA. The most recent and apparently the largest meta-analysis was conducted by Neal and co-authors in 2002. The analysis took into consideration the data of 59121 patients. By this work HO (all scores) appeared in 43% of patients who underwent total hip replacement [Neal *et al* 2002]. The meta-analysis performed earlier by Neal *et al* in 2000 covered 13 prospective randomized trials, where the patients were given NSAID-s for the primary prophylaxis of HO. These investigations showed 37% prevalence of HO if no pharmacological or radiotherapeutical prophylaxis was applied [Neal *et al* 2000a]. Owing to the clinical use of NSAID-s and in some clinics irradiation, the prevalence of HO has diminished [Bosse 1997, Puzas *et al* 1989]. Reasons for the improvement have also been advancing surgical techniques and accumulating experience of total hip replacement, which have resulted in lesser tissue damage during the surgery [Nilsson & Persson 1999].

Prevalence of HO has had large variation in different investigations. By the different data the rate of HO in the postoperative period without primary prophylaxis has been 8–90% [Ahrengart 1991, Lazansky 1973, Sawyer *et al* 1991]. Similar variability was confirmed also by Thomas [1992], in his review.

These different results can be explained by the variation of patients, variation of surgery, and postoperative treatment and the variation of rating criteria and methods.

The prevalence of HO is high, but clinical manifestations are relatively seldom detected. Usually HOs are diagnosed as asymptomatic radiological findings. Clinical symptoms have usually been connected with the more serious HO cases classified as grade III and IV on the Brooker scale. Prevalence of these serious cases was 9% as reviewed by Neal *et al* [2002]. Often a functional disorder was detected in these cases and surgery, medication or radiation became necessary [Dahl 1975, Riska & Michelsson 1979, Abrahamsson *et al* 1984, Vastel *et al* 1999, Wick *et al* 1999, Seegenschmiedt *et al* 2001].

4. Predisposing factors for HO

Considering the dangers and inconveniences associated with the application of HO prophylaxis, the question whether, when and in which patients to start prophylaxis still remains a somewhat hard decision. In order to specify the predisposing factors for HO, many studies have been conducted. There is one certain predisposing factor for HO – the male gender has been found by many authors to predispose HO [Ahrengart 1991, Ahrengart & Lindgren 1993, DeLee *et al* 1976, Duck *et al* 1992, Eggli & Woo 2001]. Similarly, the hypertrophic

type of osteoarthrosis is also one factor [Ahrengart 1991, Ahrengart & Lindgren 1993, Eggli & Woo 2001] as well as an anamnesis of HO formation [Ahrengart 1991, Duck *et al* 1992, Eggli & Woo 2001, Pedersen *et al* 1989, Sodemann *et al* 1988]. However, there are numerous other factors proposed. Moreover, most of the studies mentioned above are retrospective. Only the investigation of Eggli and Woo is based on a database consisting of data collected prospectively, but the study itself was conducted later [Eggli & Woo 2001].

5. Morphology of HO

HO formation in dynamic morphological studies resembles the histogenesis of reparative and regenerative processes of bone defects. HO has been described as a highly active tissue, with a high bone turnover and a rapid bone formation [Puzas *et al* 1989]. High turnover of bone tissue has been demonstrated as enhanced osteoblast function, being three times higher than the function of osteoblasts originating from normal bone tissue [Sell *et al* 1998]. Also osteoclastic activity has been documented to be higher than in normal bone [Bosse 1997]. Taken together, these results certainly point to increased turnover.

Presence of different zones in the forming ossificates was first demonstrated by Ackerman [1958]. He described the presence of lamellar bone and proliferating osteoblastic cells, while stroma was mainly centrally located. Ackermann proposed that during the histogenesis HO developes in a centrifugal order and thus a zonal phenomenon is generated. This is visualized on x-ray images as well as in histological preparations. However, in larger ossificates histology is not always so classical. Bosse described HOs in pressure-sores that consisted of multiple ossicles with separate organized layers in each ossicle [Bosse *et al* 1994b]. This is probably due to the multinucleate origin of the larger ossifications, resulting in formation of conglomerates. In these cases the x-ray picture as well as histologic sections resemble rather the "parquet" of small bony structures, alternating with less differentiated or fibrous connective tissue zones [Bosse *et al* 1994b]. Looking at the small ossifications forming a larger conglomerate, in detail, the zonal phenomenon is actually still recognizable. Bosse divides ossifications, according to this principle, into two main areas: the central one consisting of lamellar bone with highly differentiated cells and the surrounding, less differentiated zone [Bosse *et al* 1992].

6. Treatment and prophylaxis of HO after THA

If HO is already formed the only effective treatment is surgical removal of the ossification, which is followed by secondary prophylaxis for HO. However, to obtain satisfactory results and to avoid recurrence of HO, surgery has to be followed by secondary prophylaxis where non-steroidal anti-inflammatory drugs (NSAID), ionizing radiation or their combination are applied [Wick *et al* 1999].

In general, the surgical removal together with interpositioning of fat tissue into the potential recurrence area should be considered for non-satisfcatory outcomes (improvement efficiency 25%) [Abrahhamsson *et al* 1984]. If similar procedure is followed by secondary prophylaxis it may be even advocated in cases of less advanced HO (Brookers grade II/III) [Riska & Michelson 1979].

The most effective means for prophylaxis is ionizing radiation. In a large multi-center study on 5677 patients (a total of 5989 hip replacements) it was shown that ionizing radiation is effective if administered perioperatively starting from 24 hours prior to the operation or postoperatively during the time-interval up to 72 hours. In most patients a single dose of 7 Gy was used and HO occurred only in 475 cases (11%) if classified by Brooker's system [Seegenschmiedt *et al* 2001].

There are several studies, conducted on animal models and *in vitro,* to investigate the mechanisms of the action of ionizing radiation for HO prophylaxis [Kantorowitz *et al* 1990, Schneider *et al* 1996, Rumi *et al* 2005a]. It has been revealed, that the number of mesenchymal stem cells around the implant will decrease from which mainly an anti-proliferative effect can be expected [Balboni *et al* 2006]. Although the safety of ionizing radiation in low doses, as used for HO prophylaxis, has been demonstrated, no differences in the incidence of malignant diseases between the irradiated and non-irradiated patients were found [Seegenschmiedt *et al* 2001].

In 1975 Dahl, based on empirical findings, introduced high-dose aspirin as a means for HO prophylaxis. In later practice several NSAIDs have been approved – diclophenac, indomethacin, ketoprofen [Dahl 1975, Knelles *et al* 1997, Pritchett 1995, Vastel *et al* 1999]. It is remarkable, however, that aspirin in low doses does not exhibit the necessary prophylactic effect [Neal *et al* 2000b]. It would be useful only in large doses as described by Dahl [1975] but this leads to the safety problems. In 1992 Thomas hypothesized that, besides the surgical trauma, also inflammatory reaction following trauma plays a relevant role in the pathogenesis of HO. This mechanism could also help to explain the high efficiency of NSAIDs as prophylactic agents against HO. Moreover, it has been shown, that diclophenac exerts a direct inhibitory effect on the osteoblastic function [Sell *et al* 1999].

Neal and coworkers demonstrated in a meta-analysis that use of NSAIDs results in 57% decrease of HO risk [Neal *et al* 2000a].

The use of non-selective NSAIDs, however, leads to a variety of adverse effects, of which gastrointestinal problems may be the most serious. One study reported 15.5% of patients with a cessation of treatment due to adverse effects when they received diclophenac as a prophylaxis of HO [Jockheck *et al* 1998]. Based on the preclinical data from studies with indomethacin, celecoxib and rofecoxib, it has been demonstrated, that COX-2 inhibitors have strong inhibitory effects on the bone formation processes, especially the endochondral ossification [Katori & Majima 2000, Simon *et al* 2001]. There has been also found positive association between the mRNA levels of the cyclooxygenase-2 (COX-2) and growth factors that are inducing bone formation [Meinel *et al* 2001]. On the other hand, it has also been shown that the effect of prostaglandin E2, a common product of both COX-1 and COX-2, may be controversial from the point of view of bone induction – being able to induce activation of the osteoblastic as well as the osteoclastic proliferation [Erikssen & Kassem 1992, Kawaguchi *et al* 1995]. Osteoclasts belong to the monocyte cell lineage and they are migrating to the site of HO formation from vascular system [Bosse 1997]. Preosteoblastic and osteoblastic cells have a mesenchymal origin and their activation may be assumed to be stimulated mainly by PGE2 produced locally at the inflammatory site, apparently by COX-2 [Katori & Majima 2000].

7. Mechanisms of HO formation

7.1. Contributing conditions

As already stated above, there are a couple of predictive factors that are predisposing for HO formation. Tissue hypoxia, changes in sympathetic innervation, prolonged muscular inactivity, forced mobilization after an inactivity period, and dysequilibrium of parathyroid hormone and/or calcitonin activity have been identified as the possible contributing conditions [Shehab 2003, Vanden Bossche & Vanderstraeten 2005].

Chalmers and co-workers proposed in 1975 that three conditions are required for heterotopic ossification: an inducing agent, a suitable osteoconductive environment, and availability of osteogenic precursor cells [Chalmers *et al* 1975]. Continuing the discussion about the mechanisms in HO formation after THA Cohly *et al* have shown in a rat experiment that these mechanisms, especially cellular reactions to tissue damage and migration of osteoprogenitor cells from bone marrow, may be involved [Cohly *et al* 2003].

In the case of THA there exists marked spreading of microscopic bony fragments as well as cells from the femoral canal (including bone marrow stromal cells of mesenchymal origin). Similarly, during operation the vasculature and innervation are almost unavoidably damaged. Thus, presence of local hypoxia and disturbances of innervation are common during the endoprosthetic

replacement procedure. Also, there exists high possibility that prolonged immobilization also may play some role. It has been shown, that muscle immobilization, followed by forcible mobilization can easily lead to the HO formation [Michelsson & Rauschning 1983, Michelsson *et al* 1980]. Disturbances of humoral regulation have also been shown to induce HO in experiments with sera from quadriplegic patients [Puzas *et al* 1987]. Finally, the genetic disorders, where promoting genes for BMP-4 are constitutively expressed, are unavoidably leading to progressive ossification [Shore *et al* 2002, Hannallah *et al* 2004].

7.2. Cellular sources for HO

The origin of osteoprogenitor cells in heterotopic ossification has been widely discussed. It has been proposed that bone marrow stromal cells together with spread microscopic bone fragments may have a crucial role in heterotopic ossification after THA [Puzas *et al* 1989]. However, similar probability to be a cellular source in heterotopic ossification has been ascribed to the non-circulating connective tissue cells, representing an undifferentiated mesenchymal cell pool [Buring 1975, Owen 1980, Brighton *et al* 1992].

The idea that multipotent bone marrow mesenchymal stem cells are involved in the pathogenesis of HO is apparently as old as the observation of Friedenstein regarding the ability of bone marrow cells to direct connective tissues to osteogenic development [Friedenstein 1966]. Similarly, another important factor for HO induction – traumatic injury of muscles and formation of haematoma, which were thought to lead to the proliferation of perivascular connective tissue and turn to osteochondral development, were postulated as early as 1958 by Ackermann [1958]. This can probably be applied on humans. Finally, also periostal cells should be kept in mind as their potency to form new bone was shown by Urist and McLean in 1963.

Although the fact that in the artery walls are stem cells with potency similar to that of mesenchymal stem cells found in bone marrow was mentioned already in 1968 [Wissler 1968], it has become more topical during the last decades. It has been shown, that the perivascular multipotent cells called pericytes can under controlled conditions easily be converted into osteochondral development [Brighton *et al* 1992, Diaz-Flores *et al* 1992, Canfield *et al* 1996, Doherty *et al* 1998]. So a considerable source of the multipotent cells with a determinable osteogenic potential should be considered everywhere where vasculature can be found. Muscular and capsular multipotent cells as well as periostal cells could all be involved, as their osteogenic potential has been confirmed [Owen 1980].

Considering that formation of new bone demands proliferation of precursor cells and their differentiation into osteogenic lineage, ionising radiation has

been used to prevent HO formation after THA. Based on this knowledge also an animal model has been developed to study preventive measures for HO [Schneider *et al* 1996]. Recently, using the above-mentioned model it was shown in rabbits, that irradiation of the femoral canal resulted in lower degree of ossification as compared to irradiation of the abductor musculature. However, roentgenological multirater assessment, based on the modified Brooker's classification, was used where the mean difference between the two groups with value of 0.575 (95% CI=(0.323, 0.827)) was recorded, which was statistically significant ($p < 0.02$) [Rumi *et al* 2005b].

In order to verify the hypothesis about the main role of the stem cells and factors originating from the femoral canal, in this dissertation a heterotopic ossification model was developed. The method, using the osteoconductive matrix or osteoconductive matrix and osteoindutive protein together, allows or completely restricts the access of the femoral canal cells to the site of heterotopic bone formation, which in detail is described in the chapter "Material and methods".

7.3. Growth factors in HO

Bone morphogenetic proteins (BMPs) were discovered by Urist in 1965. These factors have the capacity to stimulate the differentiation of mesenchymal stem cells into the direction of osteochondral development. BMP-2, -4 and -7 are growth factors with certain positive effects on the bone formation [Rosenzweig *et al* 1995, ten Dijke *et al* 1994]. BMP-2 was predominantly expressed in chondrocyte-like cells [Tanaka *et al* 2001]. A high expression of BMP-2, BMP-4 and BMP-7 has been demonstrated also during ossification in the yellow ligaments [Hayashi *et al* 1997]. Considering the presence of endochondral type of ossification of the yellow ligaments [Okuda *et al* 2004] and the fact that similar process occurs within the borderline of mature ossifications [Toom *et al* 2003], a similar expression of regulatory substances can also be expected.

Of the 5 known types of transforming growth factor β's (TGF-β) [Grimaud *et al* 2002] three (TGF-β_1, -β_2, and -β_3), are identified in mammals [Roberts & Sporn 1990]. Their participation in regulation of bone formation is well known; osteoblasts have a high expression of TGF-β receptors [Robey *et al* 1987].

Sawyer and co-workers found, that the TGF-β content in HO was 6.8 times higher than the content in normal bone in 6 age-matched patients [Sawyer *et al* 1991]. Although the TGF-β's are known to strongly stimulate the bone formation, these substances are devoid of osteoinductive properties in human cells [Solheim 1998]. In non-human primates the TGF-β may induce bone formation, but its effect in rodents is similar to that in humans [Matsaba *et al* 2001].

It is documented, that the subtypes 1, 2 and 3 of TGF-β are expressed in ossifying human atherosclerotic lesions and are located mainly in cells associated with calcification. At the same time the TGF-β_2 mRNA has been found to be very highly expressed in giant cells associated with calcifications [Jeziorska 2001]. For example, this finding is corroborated by the findings that transgenic mice over-expressing TGF-β_2 in bone are characterized by increased activities of osteoblasts and osteoclasts but impaired matrix mineralisation by osteoblasts [Erlebacher & Derynck 1996]. Different expression of different subtypes of TGF-β during bone formation has been described by other authors [Horner *et al* 1998]. If TGF-β is added to the forming bone, the process is accelerated, but there is also evidence, that alkaline phosphatase activity is inhibited by TGF-β in rat [Joyce *et al* 1990]. Modulating effects of TGF-β_1 and TGF-β_2 on the BMP-2 effects during skeletal development have been documented for the mouse [Lyons *et al* 1989] and the chicken [Chen *et al* 1991]. Later it was also shown that TGF-β_3 was acting in sequential manner with BMP-2 in regulation of cartilage differentiation in chick limb formation [Roark & Greer 1994].

It has been shown that these growth factors can modulate each other's effects in human cells *in vivo* and *in vitro*. For example, recombinant human TGF-β_2 (rhTGF-β_2) induced the proliferation of human bone marrow stromal cells and increased their collagen I production *in vitro*, whereas BMP-2 promoted their differentiation into osteoblastic phenotype. At the same time increased concentrations of rhTGF-β_2 reduced the activity of alkaline phosphatase induced by recombinant human BMP-2 (rhBMP-2), which suggest their sequential effects during bone formation [Fromigue *et al* 1998].

AIMS OF THE INVESTIGATION

1. To assess the incidence and severity of HO after total hip arthroplasty and to determine the preoperative conditions predisposing for HO [original publication I]
2. To estimate differences in HO incidence depending on the use of different classifications to propose a classification system with higher reproducibility [original publications II and III]
3. To calculate the value and sources of errors appearing in assessment of HO on plain x-rays and to establish whether application of computer-assisted measurements of HO can significantly improve the preciseness of HO assessment [original manuscript IV]
4. To describe the morphology of HOs in a dynamic manner and to reveal changes of the expression of some osteoinductive growth factors (BMP-2, TGF-β_2 ja TGF-β_3) in ossifications [original publication V]
5. To reproduce HO formation in a rat model in order to investigate the source of osteoprogenitor cells [original manuscript VI]

MATERIAL AND METHODS

Table 1. Summary of studies performed for present doctoral dissertation

Type of study	Study site	Type of subjects	Number of subjects	Publication/ manuscript
Case-control study	UT	Patients	178	I: Int Orthop – 2001
Prospective analysis of reliability of HO classifications	UT	Patients	111	II, III: Estonian Physician – 2003, Int Orthop – 2005
Analysis of sources of error in HO assessment process	UT	Digitized x-ray images	28	IV: manuscript
Clinical, histological and molecular biological study	Um, UT	Tissue samples, patient data	19	V: Calcif Tissue Int – 2007
Experimental animal study	UT, Um	Rats	20	VI: manuscript submitted to Acta Orthopaedica

UT – University of Tartu, Um – Umeå University

1. Determination of the incidence and severity of HO in our clinic [I]

The data of 178 patients (66 men and 112 women) who underwent THA in the Clinic of Traumatology and Orthopaedics, University of Tartu, between 1995 and 1996 were reviewed retrospectively. All patients were treated prophylactically with NSAIDs. All patients also received antithrombotic prophylaxis. In all cases the posterolateral operative approach was used.

The preoperative anteroposterior radiographs as well as the radiographs taken on the 1st postoperative day and at 3, 6, 9 and 12 months postoperatively were assessed. HO was classified according to the Brooker classification into stages I–IV, of which stages III and IV were considered clinically and functionally significant [Brooker *et al* 1973].

2. Assessment of the influence of the classification system on the incidence of HO [II, III]

One hundred and eleven patients who underwent THA in the Clinic of Traumatology and Orthopaedics at the University of Tartu during 2002 were included in this study. There were 65 female and 46 male patients with an average age of 67.6 years and 62.7 years, respectively.

Frontal plane x-rays images were taken immediately after the operation and at 12–14 months. Four investigators evaluated the x-rays images: two were consultant surgeons and two were trainees of orthopaedic surgery. The assessment was performed single blinded and hence the investigators had no access to other analyses, and were not allowed to discuss the evaluation.

3. Determination of the predisposing factors [I]

The data of 178 patients (66 men and 112 women) who underwent THA in the Clinic of Traumatology and Orthopaedics, Tartu University, between 1995 and 1996 were reviewed retrospectively. The reason for THA was degenerative arthritis in 160 cases, rheumatoid arthritis in nine cases and trauma of the hip in nine. All patients were treated prophylactically with NSAIDs up to 30 days postoperatively. All patients also received antithrombotic prophylaxis with 0.3 ml (2500 IU) fractioned heparine (Fraxiparine) daily. The prostheses used were all cemented; Lubinus IP in 120 cases, Lubinus Sp II in 51 cases and the Link Dysplasia hip prosthesis in seven. In all cases the posterolateral approach was used.

The data collected included age, gender, diagnosis, surgery on the ipsi- and contralateral hip performed before or after THA, operating time, type of anaesthesia, blood loss and preoperative treatment with NSAIDs. Also, the preoperative anteroposterior radiographs were reviewed, which included the assessment of osteophytes and the site of subchondral sclerosis. Dysplastic hip joints were included as a specific category. With regard to osteophyte formation the patients were divided into three groups: (1) absence of osteophytes, (2) moderate osteophyte formation and (3) marked osteophyte formation with lateral subluxation of the femoral head.

4. Assessment of the sources of error in the diagnosing process [IV]

28 patients in whom total hip replacement was performed during 2002 year in the Department of Orthopaedic surgery of Tartu University Hospital were recruited into the study. All these patients had a roentgenological diagnosis of HO.

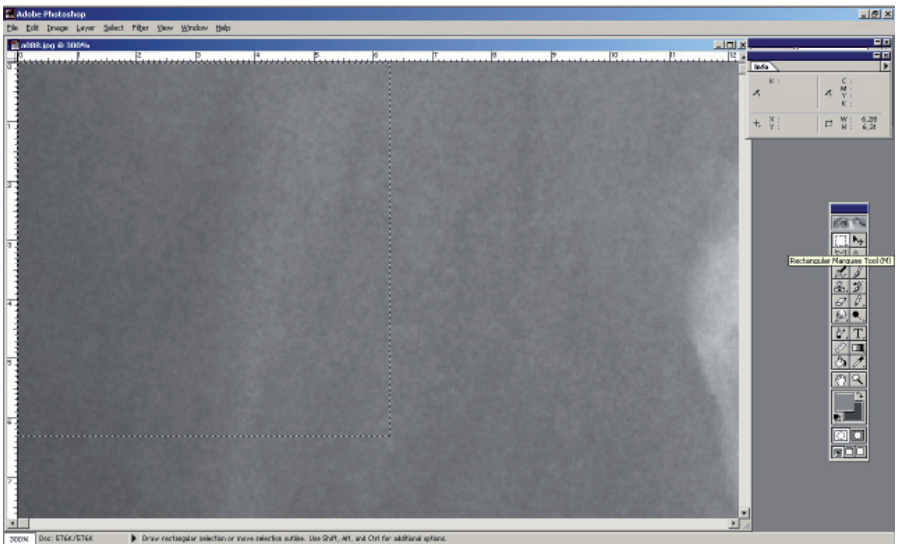
Frontal plane (plain) x-ray images were used for assessment. The x-ray was taken immediately after operation in the recovery room and 12 to 14 months later during follow-up. All the images were digitalized and all the assessments were performed using the same views of the images.

We recruited into study several investigators, who performed several observations at different times. This made it possible to find the value of technical error arising from the use of the dispersion model and, considering this calculated value, to find by estimation of remaining variation, the error arising at the time of making the diagnosis. One of possible sources of errors could be the qualification of examiners. To minimize qualification related errors together with technical errors, six investigators were recruited as follows: two of the examiners were orthopaedic surgeons, two were radiologists and two were morphologists. This allowed to differentiate between the qualification related error and personal biases. Estimation was performed in two sessions with an interval of 3 months. During the one session investigator detected the borders of ossification and marked them up by the use of computer program in triplicate in consecutive manner and averaged. Technical and diagnostic variations between the investigators were also estimated. This gave a possibility to calculate technical errors and diagnostic errors between the examiners. Two different sessions were designated for calculation of the intra-examiner error of HO diagnosis. As a blinded study requires, the investigators were not allowed to discuss results or to share opinions.

X-ray images were digitalized and Adobe Photoshop version 5.0.2 was used for estimation of the extent of HO. For the calibration of x-ray images, known isotropically projected (bilaterally symmetric) measures of the total hip prosthesis (neck diameter of the femoral component and length of the femoral head edge projection) were used. The investigator made each calibration from triple measurements of the two constant measures of the endoprosthetic head and this was achieved by applying the “measure tool” (Figure 1). The result of calibration was the known area of one pixel.

Ossifications were delineated using the “polygonal lasso tool” (Figure 2A), and were marked in different colour (Figure 2B) not presented on the gray-scale digitalized pictures. All coloured areas were measured using the “histogram” function (Figure 2C). The procedure was repeated three times and averaged. A

A



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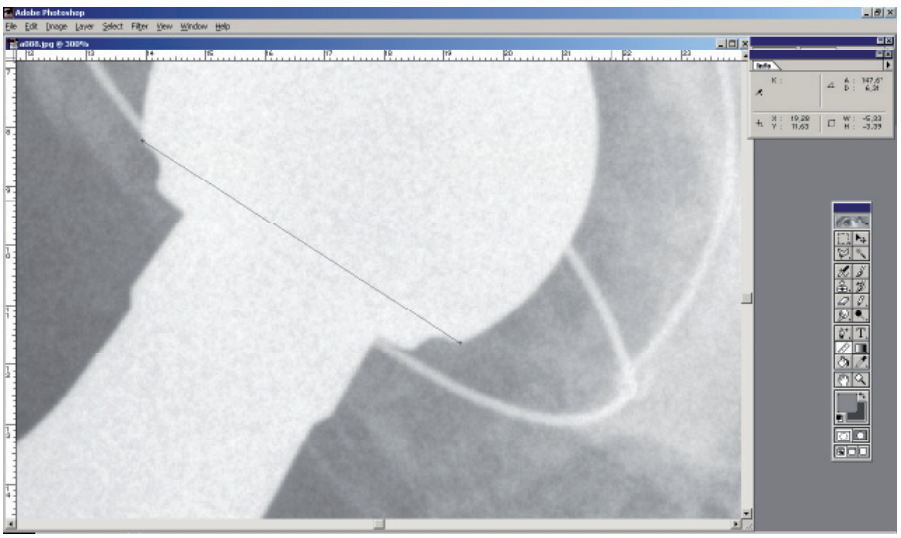


Figure 1. Calibration procedure for digitalized planimetry analysis. Explanations in the text.

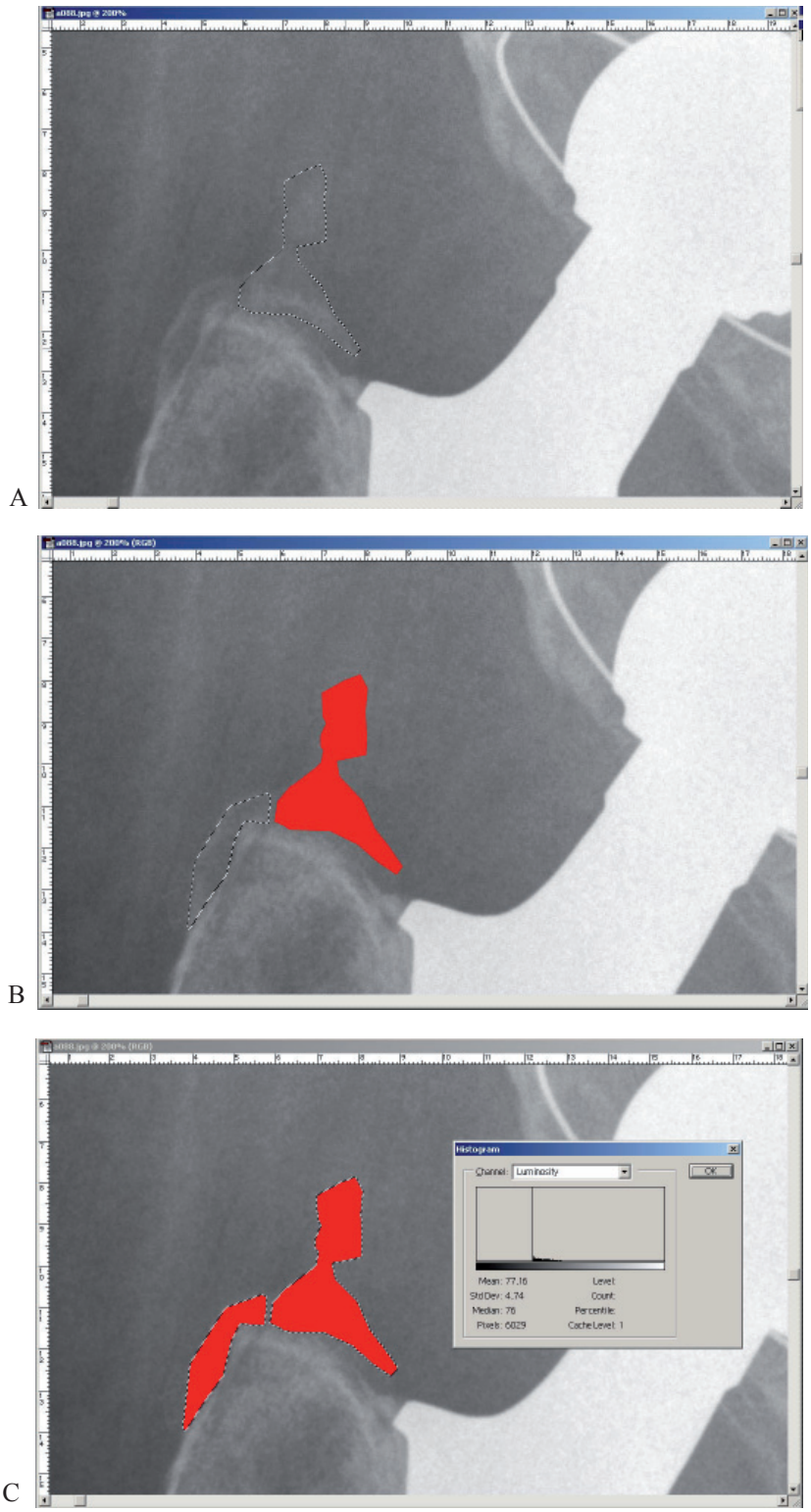


Figure 2. Calibration procedure for digitalized planimetry analysis. Explanations in the text.

similar measurement procedure, apart from the picture calibration, was repeated by each investigator 12 weeks later.

After entering the numerical measurements into the computer-based spreadsheet, the areas of ossifications for each measurement were automatically calculated and converted to the classification system proposed earlier (Schoellner *et al* 2000].

5. Obtaining patient samples for investigation of pathogenetic processes in HO [V]

Investigated subjects.

Patients undergoing endoprosthetic revision surgery due to aseptic loosening, quiescent endoprosthetic infection or heterotopic ossifications between 2001 and 2004 were invited to participate in this study. Patients with apparent tissue changes related to active endoprosthetic infection, as well as those having any rheumatic or systemic disease of connective tissue were excluded. One patient was excluded from final analysis due to the extensive period (34 years) elapsed between the HO induction and sample harvesting. The sample of HO from this patient revealed a marked osteoporosis of HO as confirmed by histomorphometric analysis.

An age and diagnosis matched control-group was enrolled according to the inclusion criteria, in the study-group. Enrolment was voluntary and all patients gave their informed consent.

Sample harvesting and preparation.

Samples of HO and control samples: fibrous tissue from the hip joint capsule and orthotopic bone from the dissected femoral neck, were harvested during revision prosthesis surgery. All these samples would otherwise have been disposed of.

Overall 7 HO samples were harvested from 7 patients. Based on reports from literature [Puzas *et al* 1989] the formation of HOs generally takes place during the first 12 months, after which time period the size of ossifications increase at a slower rate. Final size is achieved approximately during the second year after the intervention [Garland 1991, Hierton *et al* 1983, Puzas *et al* 1989]. We therefore divided the ossifications into two groups: immature and mature ossifications. There were 3 patients (1 male and 2 female) with immature HO induced 6–17 months earlier and 4 patients (2 males and 2 females) with mature HO induced 3–9 years earlier.

The control group consisted of 12 patients (4 male and 8 female). All patients except one male, who had developed necrosis of the femoral head, were operated due to coxarthrosis for idiopathic causes.

The average age of the patients in the group of immature HO was 40.3 years, in the group of mature HOs 55.5 years and in the control group 55.8 years.

HO samples intended for gene expression analysis were repeatedly rinsed in normal saline and placed in the RNA preserving medium "RNAlater" (Ambion) and HOs were macroscopically separated from adjacent tissues. Samples were then flash-frozen in liquid nitrogen (–70°C) until final dissection under a stereomicroscope.

A new dissection method was developed in order to study the spatial changes in different parts of the HO. This consisted of microscope guided dissection of different parts of HO according to the histologic picture of the adjacent area and their later use for mRNA extraction and measurements of gene expression on the mRNA level.

Final sample dissection was performed according to the histological findings of the adjacent part of the sample collected for histologic analysis. Immature HOs were dissected according to the three developmental zones (Figure 3) described in chapter "Results and discussion". Samples of mature HOs were dissected and divided into three parts according to the borders of formed bone, fibrous cartilage and surrounding compartment.

The separation procedures were performed in a dissection chamber at –68°C (using solid carbon dioxide). Pieces from the border of two different zones were discarded. Only pieces where the type could be identified by hardness and visible color were collected for further investigation. Collected samples of different types of tissue were stored –70°C until the RNA extraction.

6. Animal model of the hip region HO [VI]

6.1. Experimental animals

Twenty adult male Wistar rats at the age of 9 months (body weight 500–600 g) were used in this study. They were housed in standard cages with a 12 h-light/dark cycle and a constant temperature of 21°C with access to water and standard dry-food pellets ad libitum. Animal care and management, surgical protocol, and preparations followed the routines stated by FELASA. The Animal Ethics Committee at the University of Tartu approved this study.

6.2. Operative procedure and implantation technique

Animals were anesthetized with isoflurane (Forane®, Baxter, International Inc.) by inhalation. Antibacterial prophylaxis was performed using a single intramuscular dose of ampicillin prior to the operation. Analgesia was provided before the operation and during 72 hours postoperatively using morphine

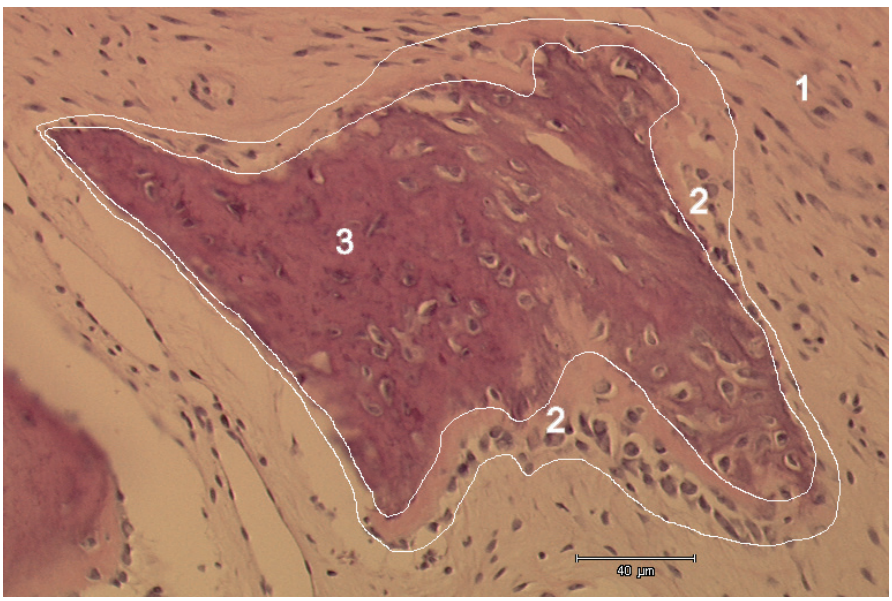


Figure 3. Three zones in immature HO: 1) zone of cellular proliferation, 2) zone of osteoid formation and 3) zone of formed bone. White lines indicate the approximate cutting lines during the separation of the immature HO into three zones, H&E staining.

sulphate. No anti-inflammatory drugs were applied. Technically, 12–14 mm incision was made over the great trochanter. Transgluteal approach was used to reach the posterior part of the hip joint capsule. While *gluteus maximus* muscle was retracted, gluteus medius was pinched with a standard vascular clamp with a width of 3 mm to produce muscular damage. Bilateral femoral capsulotomy was performed and an ordinary implant of beta-tricalcium phosphate (ChronOS™ Block, Mathys Medical Ltd, Osteosynthesis, Bettlach, Switzerland) with the size 3.3 x 3.3 x 3.3 mm and with the volume of 36 mm^3 and interconnected porosity of 70% (being theoretically able to contain approx. 25 mm^3 liquid) was implanted into the capsulotomy wound. In half of the animals, the implants were immersed in a solution of rhBMP-2 (prof Walter Sebald, Biozentrum der Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany) giving 12.5 micrograms/20 microliters per implant. Control implants were immersed in vehicle (phosphate-buffered isotonic saline). In both groups, the implant on right side was exposed to the cells originating from the femoral canal. For that purpose the femoral canal was opened just slightly medial from the tip of the great trochanter. A conic reamer with a maximal diameter of 1.8 mm and an electric drilling device were used for obtaining the aperture. Canal opening was completed manually with a troacar of 1.6 mm in diameter. No effort was made to remove the tissue remnants after the opening of the canal. On the left side the femur was left intact and care was exercised to avoid any periostal injury during the capsulotomy procedure.

Following the protocol 4 experimental groups were formed: samples from group 1 animals (with osteoconductive matrix) were divided into group 1A – samples from right side with open femoral canal and group 1B – samples from the left side where the femur was intact. Similarly the samples from group 2 animals (with osteoconductive matrix and osteoinductive rhBMP-2) were divided into group 2A – samples from right side with the open femoral canal and group 2B – samples from the left side where the femur was intact (see Figure 4).

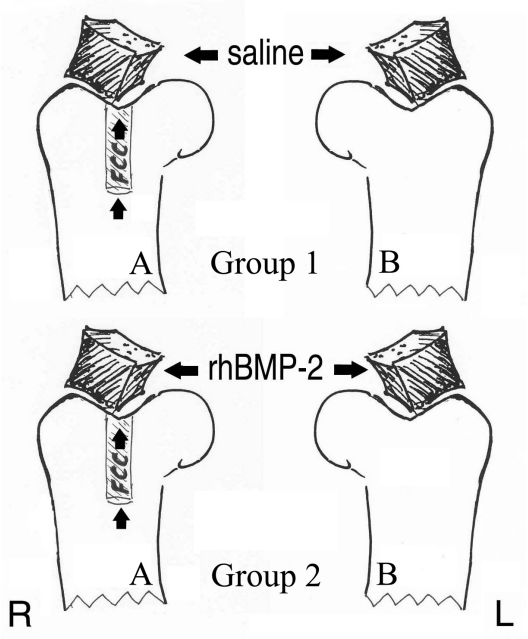


Figure 4. Experimental design for animal model.

R– right side; L – left side; FCC – femoral canal cells; rhBMP-2 – recombinant human bone morphogenetic protein-2

6.3. Euthanasia

Rats were decapitated under sedation with isoflurane 21 days after operation.

7. Histological methods [V and VI]

7.1. Human samples

Samples for histology were rinsed with normal saline and fixed in neutral buffered 4% formaldehyde solution. One part of the harvested samples were decalcified with EDTA according to the method of Sanderson *et al* [1995] and then dehydrated by alcohol-chloroform solutions and embedded into paraffin.

7.2. Experimental samples

Samples from animal experiments were fixed in neutral buffered formalin. After decalcification [Sanderson *et al* 1995] histological sections were made following the principle of systematic uniform random selection [Gundersen 2002].

Hematoxylin-eosin, AZAN and metachromatic staining with toluidine blue were used in both types of samples.

8. Histomorphometric methods [V and VI]

8.1. Human samples

Surfaces from both sides, which were adjacent to the part of the samples used for determination of growth factor expression, were intended for histomorphometric analysis. Starting from the first intact section (usual trimming depth 50 up to 200 μm) two section-pairs with the thickness of 5 μm were randomly selected from two blocks with the step of 25 μm. Photographed fields were smoothly fractionated, as described by Gundersen [2002] and the number of counting points was at least 50 for each slice. The physical dissector method as described by Sorensen in 1991 [Sorensen 1991] was applied for counting the cells allocated on bone surface and the type of bone surface was identified**.**

8.2. Experimental samples

Osteoid surfaces were counted in parallel on AZAN and on osteocalcin/ metachromatic stained sections and cellular density was estimated on AZAN and on hematoxylin-eosin stained sections. Mean values were calculated and used for final analysis. To express the osteoid surface density, following ratios were used: the ratio of osteoid surface to periostal surface (OS/Ps) and the ratio of osteoid surface to endostal surface (OS/Es). Applying them for HOs the outer bony surface of ossicles was considered as periostal and inner bony surfaces as endostal. Both ratios indicate the relative amount of the osteoid on respective surfaces.

Sections with the thickness of 5 micrometers for surface analysis according to the Cavalieri's principle [Gundersen *et al* 1988b] and sections with the thickness of 40 micrometers for the surface/cell counting were collected systematically after every 200 micrometers. Those sections were intended for systematic uniform random selection [Gundersen 2002], which yielded 7–8 sections of each sample for final analysis. Sections were stained with azan and toluidine.

Cell counting was performed according to the physical dissector principle [Gundersen *et al* 1988a] with the light microscope "Olympus BX51" and analysis software "Cast 2". Applying Cavalieri's principle [Gundersen *et al* 1988b] the relative and absolute volumes of different types of tissue in heterotopic ossifications were calculated using a correction factor to eliminate the tissue shrinkage effect. Also, in the region of formed bone, surface density and the proportions of eroded and osteoid surface were measured. All morphometric abbreviations used in our studies are based on the standardized bone histomorphometric nomenclature [Parfitt 1987].

9. Immunochemical stainings [V and VI]

Decalcified and deparaffinized sections were treated with 0.6% H_2O_2 to inactivate endogenous peroxidase and then with 1% BSA to block nonspecific binding.

After blocking, sections of experimental samples were incubated with primary antibodies either with a mouse monoclonal antibody to osteonectin (Acris Antibodies GmbH, Germany), mouse monoclonal antibody to osteocalcin (Abcam Ltd., United Kingdom), or rabbit polyclonal anti-collagen type I antibody (Research Diagnostics Inc., NJ, USA) for 2 hours at 4ºC. Sections of human samples were incubated with mouse monoclonal [OC4-30] antibody to osteocalcin (ab 13418), for 2 hours at 4ºC or with rabbit polyclonal antibodies to TGF-$ß_1$ (ab27969), TGF-$ß_2$ (ab15539), TGF-$ß_3$ (ab15537) or BMP-2 (ab14933) overnight at 4ºC (all antibodies were produced by Abcam Ltd., United Kingdom).

Visualization of the primary antibodies was performed using the commercial kit "Strept ABComplex/HRP Duet Mouse/Rabbit system" (Dako Cytomation Denmark A/S, Denmark), which uses the goat anti-mouse/anti-rabbit secondary antibodies and DAB+Chromogen (Dako Cytomation, USA) for substrate; metachromatic staining with toluidine blue was used for background.

10. Total RNA extraction [V]

For RNA extraction, bone tissue was crushed using the metal bone-crushing device maintaining low temperature of pieces by using liquid nitrogen around the crushing tube. After sample crushing the pieces were warmed up to 4°C in order to eliminate the free bone marrow cells, rinsed thoroughly with buffered saline and dried. The sample was then homogenized in a tissue homogenizer Ultra-Turrax T25 (Janke & Kunkel, IKA®-Labortechnik, Germany) in Trizol reagent (Invitrogen life technologies, Stockholm, Sweden). The RNA extraction

procedure was then conducted according to the manufactures instructions. The total RNA concentrations were measured spectrophotometrically and the RNA integrity was controlled by an agarose gel electrophoresis. The absence of DNA contamination was assessed by PCR.

11. Semi-quantification mRNA expression [V]

1 µg of the total RNA was used for semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) with gene-specific primers. The RT-PCR was performed using the commercial kit Access RT-PCR System (Promega, Falkenberg, Sweden) according to the manufacturer's instructions.

Primers for the RT-PCR were designed using the Oligo Primer Analysis Software Version 6 (MedProbe, Oslo, Norway). Primers were ordered from CyberGene AB, (Huddinge, Sweden). The primer sequences were as follow: human β-actin (product length 427 bp) forward primer 5'-GGCACCACACCTTCTACAAT-3'; reverse primer 5'-GCCATCTCTTGCTCGAAGT-3'; human BMP-2 (product length 330 bp) forward primer 5'-GCAAAGAAAAGGAACGGACA-3'; reverse primer 5'-GTCTCTGTTTCAGGCCGAAC-3'; human TGF-β_2 (product length 407 bp) forward primer 5'-TGCCTGAACAACGGAT-3'; reverse primer 5'-GGTCTGTTGTGACTCAAGTCT-3'; human TGF-β_3 (product length 409 bp) forward primer 5'-TGCTGAACTTTGCCACGGT-3'; reverse primer 5'-TCTGCTCGGAATAGGTTGGTTCT-3'.

The β-actin primer was used as internal control because β-actin is supposed to be constantly expressed in the mesenchymal tissues as well as unaffected by the activity of TGF-βs in osseous tissues [Pisano *et al* 2003]. PCR reactions were optimized according to the standard procedures [Saiki *et al* 1988] in order to achieve linearity for all primer pairs under the same general conditions. The PCR products were labeled with ^{32}P-ATP (Amersham Biosciences, Buckinghamshire, England) during the synthesis reaction and separated on a 1.5% agarose gel. The bands and some blank gel-pieces of equal size were cut out from the gel and dissolved in 4 ml scintillation fluid (UltimaGold, Packard Bioscience, Groningen, The Netherlands) overnight. The blank gel from the lanes was used as control to set the zero values. The radioactivity was measured in a Wallac 1409 Liquid Scintillation Counter (Wallac Oy, Turku, Finland) and the relative expression levels of mRNA to β-actin mRNA were calculated.

12. Statistical methods

The reliability of different classification systems was assessed using the proportion of the agreeing observed pairs of the maximum possible agreeing pairs using Cohen's kappa coefficient, which is a measure of the chance-corrected agreement [Cohen 1960]. Therefore, the reproducibility of the different classifications is reflected. Statistical software package R, version 1.9.0 for Windows [Dalgaard 2002] and its contribution package "concord" were used for statistical calculations. Statistical significance was considered significant at the conventional 5% level.

For determination of the predisposing factors the statistical analysis of parametric data was performed by ANOVA, and for the analysis of non-parametric data and ranker parametric data the chi-square test was used with $P<0.05$ as the level of significance.

For determination of the sources of error during HO diagnosing process the dispersion model was developed by use of computer program STATISTICA. Statistical significance was defined as $p<0.05$.

Statistical calculations of histomorphometric data were performed for different groups in the human study using ANOVA analysis. In the remaining calculations in the human study as well as for the experimental material, t-test was used both for paired and unpaired data.

In order to compare the relative values of expressed mRNA in ossifications and control tissues, the quartiles were calculated and differences between different sample groups were compared using Mann-Whitney analysis. In order to diminish the variation in intra-sample comparison and to assess the temporo-spatial dynamics of the gene expression inside of each HO growth factor, levels were normalized to the zone of formed bone and ANOVA with the *post-hoc* testing was applied for revealing statistical significance between different zones.

RESULTS AND DISCUSSION

1. Incidence and severity of HO [I]

The distribution of patients according to the grade of HO is shown in Table 2.

Table 2. Distribution of patients by the Brookers grade [Brooker *et al* 1973] of HO

HO grade by Brooker	Female	Male
0	88	33
I	19	15
II	2	5
III	2	7
IV	1	6

The overall incidence of HO 1 year after THA was 32% (57 cases). In men the incidence was 50% (33 cases) and in women 21% (24 cases); and thus the relative risk of the development of HO in males was 2.3 times higher than in females ($p<0.001$). The incidence of significant ossification in men was 4.5 times higher than in women ($p=0.026$). The average age of men without HO was 62 years and that of women was 63 years. These values were somewhat lower than in patients with grade I–IV HO (average age of men 65 years and of women 66 years) but this difference was not statistically significant.

The incidence of different localizations of acetabular subchondral sclerosis, the extent of osteophyte formation and the incidence of joint dysplasia did not differ between the patients with and without HO. Similarly, the distribution of patients by diagnosis, amount of transfused blood and type of anaesthesia seemed not to affect the presence of HO. The incidence of HO was increased if the length of the operation was more than 100 min ($p=0.041$).

Nine patients of those with HO and 22 patients of those without HO had undergone previous surgery to the ipsilateral hip, thus without an increased incidence of HO later. A significant difference in incidences was found in case of previous surgery other than THA to the contralateral hip. The risk of the development of HO in these patients was increased by a factor of 2.3 ($p=0.042$). The risk of the development of HO was also increased in patients who had undergone contralateral THA – the risk of the HO was increased in these patients by a factor of 4.9 ($p=0.003$). Contralateral THA, however, did not increase the risk after ipsilateral THA.

In patients, who had used preoperatively NSAIDs, the incidence of HO was lower. In those patients who did not receive preoperative NSAIDs the risk of developing HO was higher by a factor of 2.2 ($p=0.012$). There was a statistically significantly higher incidence of HO in patients with non-0 blood

group in the AB0-system and the risk was three times higher than in those with the 0 group (p=0.003).

2. Evaluation of classification reliability and proposal of a new classification for HO assessment [II and III]

We found the incidence of HO being related to the classification system used (Table 3). Reliability of the tested classifications is presented in Table 4.

Compared to the previous studies [Della Valle *et al* 2002, Wright *et al* 1994] our Kappa values are relatively high. One possible reason for the apparent increase of inter-observer reliability is the fact that analyses with multiple classifications were performed sequentially on the same patients.

Although we were able to increase the reliability of Brooker's classification by limiting the number of classes, this action is arbitrary and may diminish the precision of this system. Furthermore, this action does not eliminate two problems mentioned by Wright co-authors [1994]. The most common of these problems deals with isolated ossifications that have such a large size that it occupies most of the distance between the opposite bone surfaces. In our study, there was a similar problem in 5.9% cases (4–9 cases recorded by different observers). Such ossifications may potentially cause clinical symptoms, while Brooker's system classifies them as class I. Della Valle's system considers the size of such ossifications and places them in the intermediate class (B).

Since Brooker's classification is currently most widely used, we decided to create a new system, which is based on any of the other systems showing higher reliability than the Brooker's classification, so that the result will be comparable to the results of previous studies.

Arcq's classification showed the highest proportion of agreement, but lacked precision. Almost all cases of HO in our study group were graded "1", which partly accounts for the high Kappa value. DeLee's system grades the extension of HO on the basis of a different principle. Therefore, we decided to combine Brooker's classification with Della Valle's system to provide adequate inter-observer reproducibility and to improve consistency in classification of significant HO [Della Valle *et al* 2002]. Della Valle's system is basically not comparable to previous studies because of its different criteria but is easily convertible to both of its parent systems.

Our classification considers three levels. The first resembles the system of Della Valle and co-authors using classes A, B and C. Reproducibility of this part of the classification is the same as in Della Valle's system. Our assessment gave a kappa value 0.881.

The second level integrates Brooker's system, and we suggest using this level for clinical work and research. Numbers 0–3 are added to the letters so "0"

marks the complete absence of ossification, “1” refers to ossifications located in soft tissues, “2” refers to marginal ossifications, and “3” indicates the presence of complete bridging, which clinically and radiologically indicates fibrous or bony ankylosis.

Table 3. Incidence of heterotopic ossification (percentage) using different classifications

System	Brooker	Della Valle	Arcq	DeLee subclasses
Observer 1	27.9	21.6	27.9	24.3
Observer 2	24.3	12.6	24.3	19.8
Observer 3	28.8	20.7	28.8	26.1
Observer 4	29.7	24.3	29.7	27.9
Average	27.7[a]	19.8[a,b]	27.7[b]	24.5

[a,b] Indicate the statistical significances.

Table 4. Kappa values of different classification systems

	System	Brooker	Della Valle	Arcq	DeLee subclasses
n=111	Kappa value[a]	0.814	0.871	0.897	0.862
	95% CI	0.72–0.91	0.81–0.93	0.76–0.96	0.80–0.99

[a]Kappa values based on all measurements.

The second level of our classification solves the problem of extensive, isolated ossifications, allocating them between classes B1 and C1 on the same basis as marginal ossification. They are thus divided on the basis of the overall free distance left between the pelvis and femur, which can be considered to be most important criterion to determine the range of motion. The first and second levels of the classification are presented and compared to the other rating systems that are based on frontal roentgenographs in Table 5.

Table 5. Comparison of the first and second levels of the proposed classification to the corresponding levels of other classifications studied

Proposed classification		Brooker's system	Della Valle's system	Arcq's system	DeLee's system
Level 1	**Level 2**				
A. Ossification absent or small	A0. Absence of ossifications	0	A	0	0
	A1. Isolated ossifications less than 1 cm in length	I	A	1	0 or 1
B. Ossifications leaving MORE than 1 cm distance between pelvis and femur	B1. Isolated ossifications at least 1 cm in length	I	B	1	1 or 2
	B2. Marginal ossifications	II	B	1	1 or 2
C. Ossifications leaving LESS than 1 cm distance between pelvis and femur or ankylosis	C1. Isolated ossifications at least 1 cm in length	I	B	1	1 or 2
	C2. Marginal ossifications	III	C	1	1 or 2
	C3. Ankylosis	IV	C	2 or 3	3

The third level of the new classification determines the localisation of the ossification. This makes our system comparable to the parts of DeLee's classification dealing with the localisation of HO. Briefly, capital letters "L" or "M" are attached to the numbers indicating ossifications on the lateral or medial side of the implant. In addition, number "2" is supplemented by lowercase letters "p" or "f", referring to the pelvic or femoral localisation, respectively.

Why was the classification, based on frontal plane x-rays, chosen? In the literature we found two articles, that demonstrated different situations, where the severity of HO can be truly estimated if at least two different x-rays (plane and lateral) are used [Moed & Smith 1996, Schmidt & Hackenbroch 1996]. These systems demonstrated appearance of HO in regions, which are not detectable on the frontal plane x-rays, but they did not influence proportions of different stages of HO. The important reason for the current study was to improve the reliability of the classification based on the frontal plane x-ray images. There were some practical reasons as well – x-ray images of the study with 400 patients who had undergone total hip replacement during 2002–2003 in the Department of orthopaedic surgery of Tartu University were available. They were participants of a larger prospective study with adequate series of plain x-rays. In the current investigation were included patients who were operated during the first year (2002) and who passed correctly follow-up.

3. Determination of the error sources in HO assessment using digitalized planimetry measurements in a dispersion model [IV]

The results are described by formula:

$$Y_{ijkl} = a_i + b_j + c_{ij} + d_{ijk} + e_{ijkl};$$

where
(Y) is measured result; (i) patient number; (j) examiners number; (k) number of session and (l) number of repeated estimation; (a), (b), (c), (d), and (e) – different functions for different arguments. So the dispersion components have the following meaning:

a_i variation arising directly from x-ray, calculated as the total average of all examiners and all observations;

b_j systematic variation of every single examiner (biased error)

c_{ij} variation arising from co-action of examiner and x-ray– describing variation between examiners

d_{ijk} variation arising as co-action of every examiner, x-ray and observed session – describing variation of examiner

e_{ijkl} variation arising from the detecting and selecting image by every examiner, so-called technical error or methodological error

The estimation of the dispersion component corresponding to every parameter is given in Table 6.

Table 6. Estimation of dispersion components and their significance.

Covariance Parameter	Standard estimate	Error	p-value	Percent of standard estimate in error component
X-ray **(a_i)**	31416	8859	0.0004	
Technical variation **(b_j)**	598	515	0.2457	5.7%
Inter-observer variation **(c_{ij})**	2693	848	0.0015	25.5%
Intra-observer variation **(d_{ijk})**	6432	769	<.0001	60.9%
Residual **(d_{ijk})**	846	65	<.0001	8.0%

The analysis demonstrates that the main part of the measured values originates from x-ray itself while the variation arising from the different observations is considerably smaller. This shows that the method of digitalized planimetry enables to estimate the HO on radiographs. More important sources of dispersion are differences between the estimations of different examiners as

well as variability between the different diagnoses/observations of the same examiner.

The largest part of dispersion of digital planimetry arose from the x-ray image itself. This indicates clearly that the method of digital planimetry shows the differences that have been found objectively on the radiographs. The remarkable part of variations arose from the observations of the same x-ray image by the same examiner at different sessions (estimation=6432; $p<0.0001$). To some extent lesser but also statistically significant was the variation between the different examiners (estimation=2693; $p=0.0015$). Systematic deviations among the examiners were not detected. This means that there was no examiner realated bias (estimation=598; $p=0.2457$). Systematic deviation was not detected if examiner-pairs with different backgrounds (orthopaedic surgeon, radiologist, morphologist) were compared.

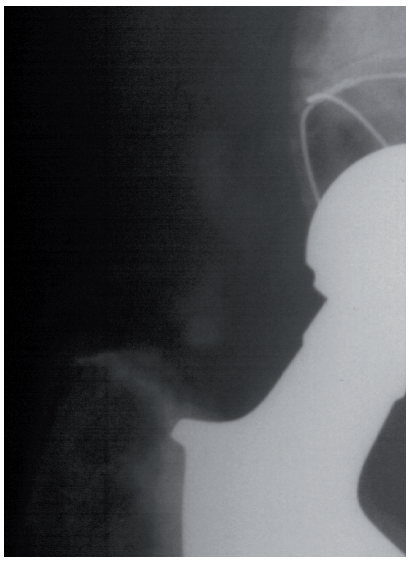
The variation arising from differentiation and selection of borders of ossificates, using the computer program Adobe Photoshop, was remarkably lower than intra- or inter-observer variation. However, the technical error had a statistically significant influence on the sum of errors (estimation=846; $p<0.0001$), but its magnitude was 10 times less than the dispersion of diagnosing process. Summarizing these results we may conclude, that the diagnostic criteria for the estimation system and skilfulness of its use play a main role in arising of diagnostic error but in this experiment the professional background was not significant.

4. Morphology of HO and growth factor expression in the bone formation zone [V]

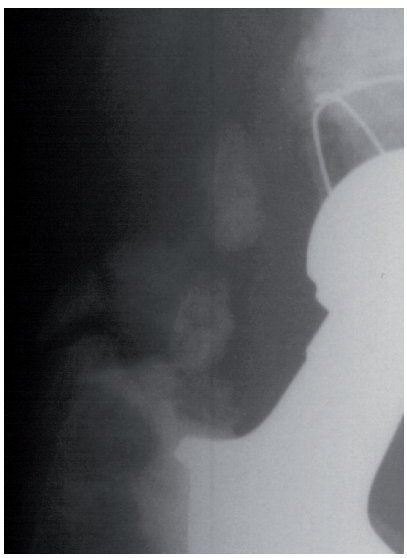
4.1. Morphology of HO

Six months after HO induction. There was one case of HO that consisted of multiple 1–4 mm ossicles formed almost completely of woven bone. Their outer surface was surrounded by numerous osteoblasts. The tissue surrounding the youngest ossification contained many star-shaped cells with multiple processes and large nuclei; i.e., their morphology resembled that of cells of undifferentiated mesenchymal tissue. The OS/Ps ratio for this particulate sample was as much as 47.2%. Almost all the bone formation was intramembranous. All those signs indicate intense bone formation.

Up to 2 years after HO induction. There were two ossifications harvested 11 and 17 months after the primary surgery. These ossifications were both similar and consisted of some small ossicles with clearly distinguishable outer surfaces and bone marrow inside. Active bone formation was still taking place on the outer surface. The bone formation was mainly intramembraneous, but also some



A



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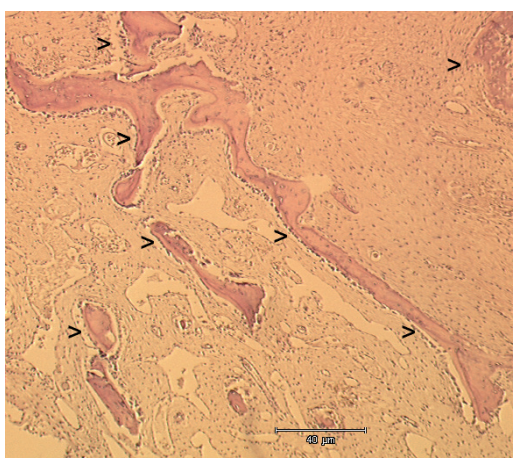

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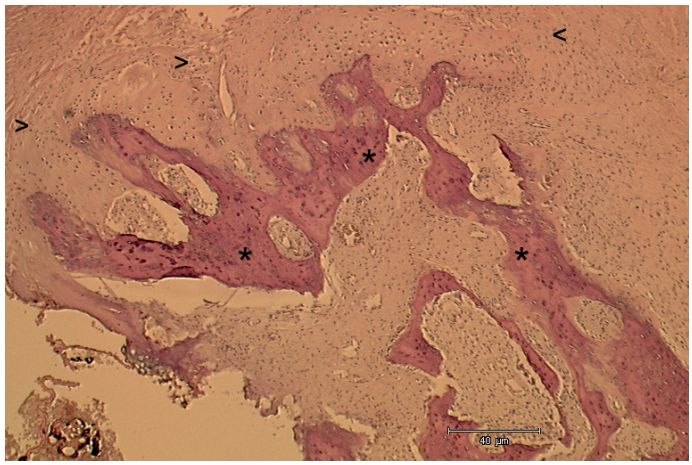

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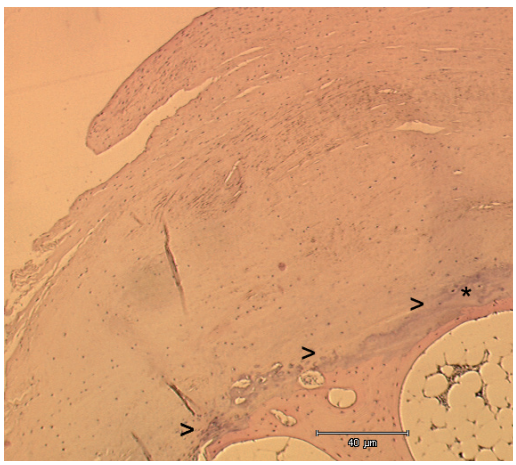

E

Figure 5. Borderline of the immature and mature HO on x-ray images and histologic pictures. 5A: X-ray photo of an immature HO taken 3 months after THA. Note laterally to the endoprosthesis an immature HO with blurry borders. 5B: X-ray of the same patient taken 11 months after THA. Note that the initially formed HO has achieved maturity, the outer border of HO has become clearly distinguishable. Also some immature HO have accrued. 5C: One example of an immature HO. Note the multiple fronts of intramembranous ossifications (arrowheads). Sample harvested 6 months after THA. 5D: Another example of histological picture of an immature HO. Note the fibrocartilage, which is partly also calcified (arrowheads) followed by initial ossification more internally (asterisk). Sample harvested 6 months after THA. 5E: Typical borderline in a mature HO. Note clearly distinguishable border between the ossificate and surrounding tissues (arrowheads). Some fibrocartilage is also present (asterisk).

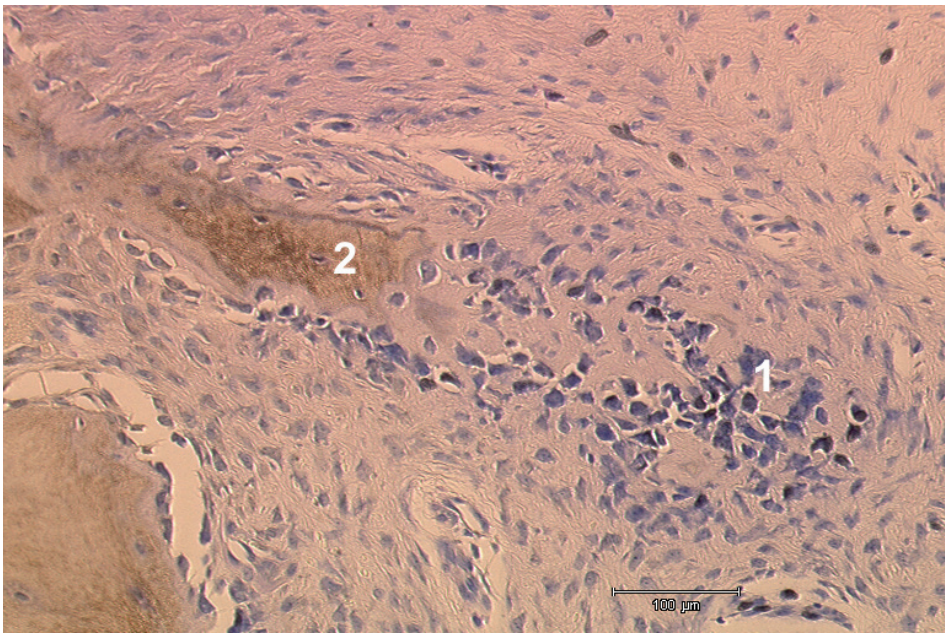

Figure 6. Formation of the bone trabecule by intramembranous ossification in the middle zone of an immature ossificate. Labels on picture: 1) osteoblasts and 2) mineralized osteoid containing osteocalcin. Immunohistochemical staining for osteocalcin and toluidine blue counterstain.

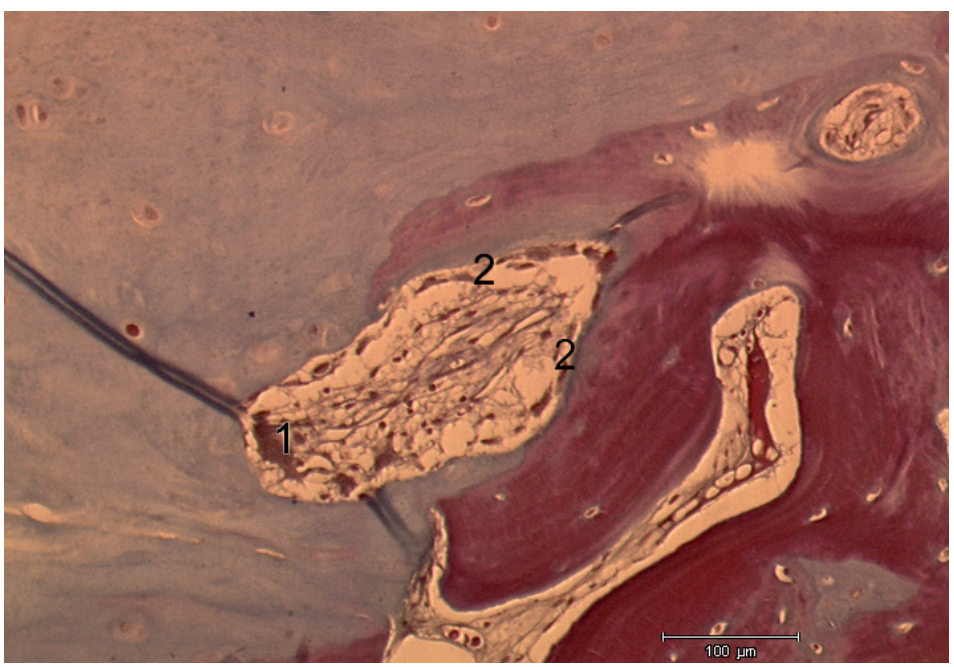

Figure 7. Bone formation in the middle zone of a mature ossificate. Labels on picture: 1) chondroclasts resorbing calcified fibrocartilage and 2) osteoblasts replacing it with osteoid. Abundance of this kind of remodelling activity is reflected also as higher amount of osteoid surface in mature HO's as compared to the control bone. Azan staining.

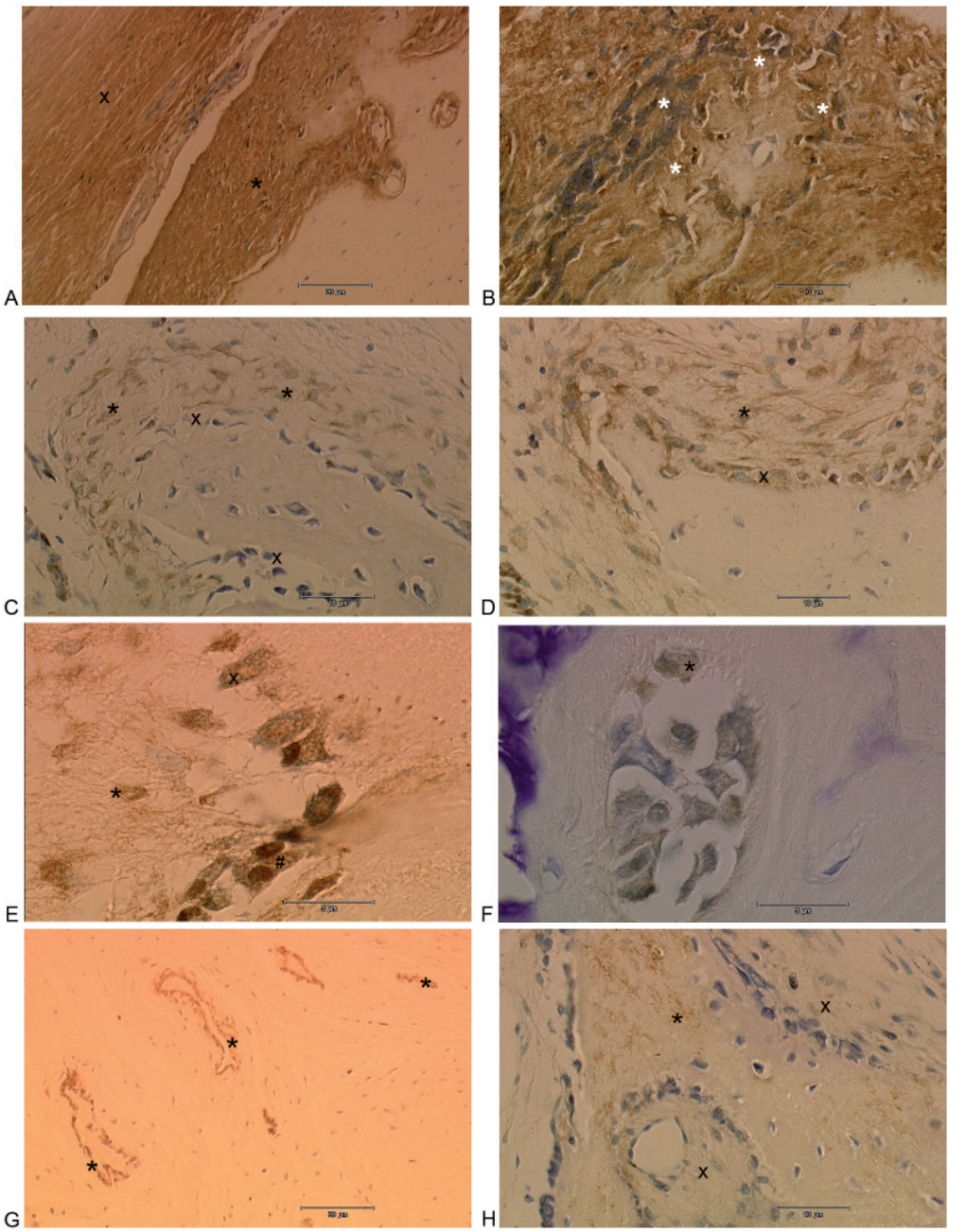

Figure 8. Immunohistochemical findings for different growth factors in heterotopic ossifications. All sections were counterstained with toluidine blue.

8A: Immunohistochemical expression of TGF-β_1 protein in bone forming zone of heterotopic ossifications (*) and in surrounding fibrous tissue (x). 8B: Immunohistochemical expression of TGF-β_1 protein in regions of osteoblastic activity (*) mainly in regions of matrix synthesis and mineralization. 8C: Immature heterotopic ossification with immunohistochemical expression of TGF-β_2. Clear signal of TGF-β_2 is detectable in the bone formation zones in preosteoblastic cells (*), but not in more differentiated osteoblasts (x). 8D: Mature heterotopic ossification with immunohistochemical expression of TGF-β_2. Signal of TGF-β_2 is detectable in the bone formation zone in preosteoblasts (*) as well as in osteoblasts (x). 8E: Immunohistochemical expression of TGF-β_3 protein is detectable in the bone formation zone in preosteoblasts (*), in osteoblasts (x) as well as in the osteoclasts (#) of the bone formation zone of immature ossifications. 8F: Remodelling zone of mature ossifications with immunohistochemical expression of TGF-β_3. Osteoblasts expressing the TGF-β_3 protein are marked with (*). 8G: Immunohistochemical expression of TGF-β_3 protein. Staining is detectable in vascular walls (*) in tissues surrounding heterotopic ossifications. 8H: Immunohistochemical expression of BMP-2 detected in matrix of woven bone (*) and in areas surrounding the osteoblasts (x) in all stages of differentiation in immature ossificates.

endochondral ossification was detected on the outer surface replacing fibrocartilage-like tissue. All three ossifications were surrounded by fibrous connective tissue. The tissue surrounding these ossifications also contained star-shaped cells with multiple processes and large nuclei, characteristic of the cells of undifferentiated mesenchymal tissue.

In *all ossifications with the interval less than 2 years between harvesting and HO induction* proliferation of low-differentiated cells with a morphology similar to that of preosteoblastic or prechondroblastic cells was noted (Figures 3, 5). All these ossificates typically had intramembranous bone formation type dominating (Figures 5, 6).

More than 2 years after HO induction. The older four ossifications were harvested 3, 6, 8, and 9 years after the primary operation and were all histologically very similar. They consisted of either one or a few ossicles where cross-sectional morphology was typical for trabecular bones: spongy area with bone marrow spaces surrounded by cortical bone. The outer surface of ossificates was mostly surrounded by fibrocartilaginous tissue. The borderline structure of mature ossifications often resembled the enthesis, as described by Benjamin *et al* [2002]. However, on the border of formed bone and fibrocartilaginous tissue, significant bone and cartilage remodeling, and thus osteoblastic activity, was detectable (Figure 7).

4.2. Bone cells and expression of the growth factors

Immunohistochemistry showed the presence of all TGF-βs in bone-forming zones of HOs. TGF-β_1 was ubiquitously expressed in HOs as well as in dense regular connective tissue (Figure 8A). Marked expression was detected also in regions of osteoblastic activity, mainly in regions of matrix synthesis and mineralization (Figure 8B). A clear signal of TGF-β_2 was detected exclusively in the bone formation zones in preosteoblasts (immature HOs) (Figure 8C), whereas during remodeling its expression was clearly detectable also in differentiated osteoblasts (immature and mature HOs) (Figure 8D). TGF-β_3 was strongly detectable in all stages of differentiation of osteoblastic cells in the bone formation zone in immature ossifications and in osteoclasts (Figure 8E). In remodeling zones of mature ossifications, expression was present, too (Figure 8F). Based on subjective visual assessment, the signal was weaker in remodeling areas than in areas of bone formation. High protein levels were seen also in the vascular walls of surrounding tissues (Figure 8G). Protein expression of BMP-2 was detected mainly in matrix of woven bone, osteoblasts, and areas surrounding the osteoblasts in all stages of differentiation in immature ossificates (Figure 8H), whereas in mature ossifications the expression was hardly detectable.

Histomorphometric analysis revealed that in ossifications both the ratios OS/Es and OS/Ps were several times higher compared to the ratios for normal bone (Figure 9). A clear difference was seen also between immature and mature ossifications, where the OS/Es and OS/Ps ratios for immature ossifications were 45.3% and 46.2% vs. 12.6% and 7.4% for mature ossifications, respectively (Figure 9). Despite different ages of mature ossifications (3–9 years), no relationship between the age of ossifications and the amount of osteoid-matrix was found.

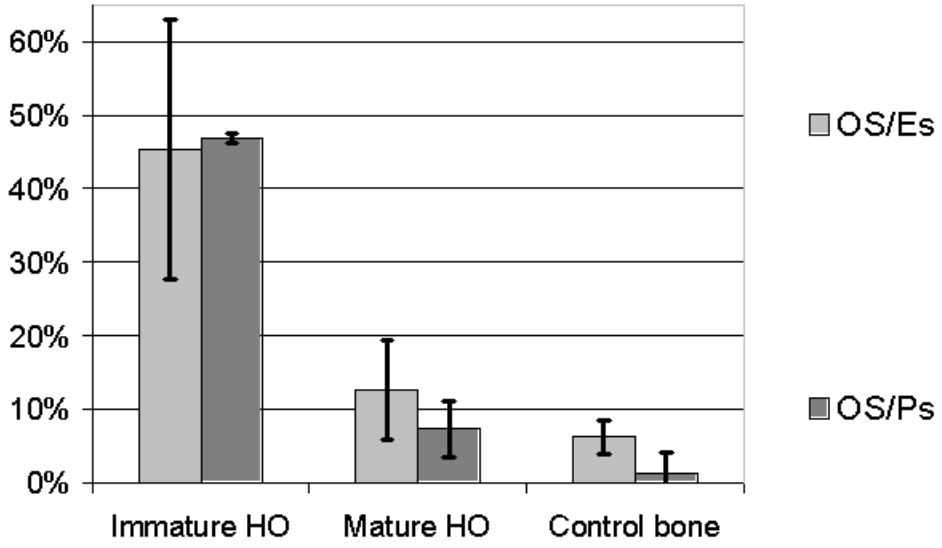

Figure 9. Ratios of osteoid surface to endostal surface (OS/Es) and osteoid to periostal surface (OS/Ps) in immature ossifications. Osteoblastic activity expressed as osteoid surface ratios are higher in immature ossifications than in mature ossifications and control samples.

The fact that matured HOs were surrounded by structures that resemble the structures of the tendon enthesis [V], which are decribed by Benjamin *et al* 2002, may be indicative of the adaptation of the organism to the presence of the HO. This hypothesis is supported by our findings of growth factor expression. The outer zone of HOs (adjacent tissue) had not only a structure similar to the structure of enthesis, but also the profile of the growth factor expression resembled to that reported earlier. Namely the high expression of TGF-β_3 and TGF-β_2 mRNA as well as immunohistochemical staining of TGF-β_2 in all stages of osteoblastic cell differentiation is similar to the profile of growth factor expression in the enthesis organ [Robbins *et al* 1997, Benjamin & Ralphs 2004].

However, a significant difference was the absence of the clear tidemark line, that is very characteristic for fibrocartilagineous enthesis [Benjamin *et al* 2002]. But this can be explained by the nature of the HO – they are heterogenic and do not fulfil a specialized function. In other words, enthesis is mediating tensile forces in a clearly determined range of directions, which causes strong shear stress at the site of insertion [Canoso 1998].

Histological findings were conformant with the clinical knowledge that HOs mature during 1-2 years [Puzas *et al* 1989] and their size will be almost constant after 2 years of development [Puzas *et al* 1989, Petty 1991].

The difference in the osteoid surface density of the outer border of ossicles was in concordance with the above findings.

Growth factor expression in HOs compared to normal bone and fibrous tissue. Expression levels of TGF-β_3 were 0.61±0.12 in the bone formation zone and 0.59±0.09 in surrounding tissue, which were significantly higher than in control bone (0.30±0.03, p= 0.003 and 0.001, respectively) but not in capsular tissue (0.43 ± 0.06).

Similarly, the level of TGF-β_2 in the bone formation zone of ossifications (0.79±0.35) was significantly higher than that in control bone (0.32±0.04, p = 0.050), whereas the difference from capsular tissue was not significant.

Levels of BMP-2 were quite similar in all groups. However, the highest expression (0.56±0.16) was detected in the zone of bone formation, which was significantly higher than the mean value in the zone of formed bone of HOs (0.24±0.02, p = 0.040). However, it did not differ significantly from control sample values.

Growth factor expression in different zones of HOs. Expression levels of growth factors were normalized to the zone of formed bone (Figure 10). All middle zones (zones of bone formation) had higher levels of BMP-2, TGF-β_2, and TGF-β_3. In the outer zone, TGF-β_2 and TGF-β_3 were higher than in the central zone (zone of formed bone).

To eliminate the influence of different systemic factors and of different intervals between induction and harvesting of different HOs, we decided to compare also gene expression levels in different parts of the ossifications and to use these data for comparison with the age- and gender-matched control samples from the femoral neck. Patients with immature ossifications were younger than patients with mature ossifications and subjects of the control group, but this difference can be considered unimportant as there has been shown absence of age- or gender-related differences in the expression levels of these growth factors in bone samples from the iliac crest [Bunger *et al* 2003]. Thus, spatial changes occurring during HO formation within the three zones give a clearer picture of possible temporal changes during HO formation and eliminate related intersample variation of gene expression levels.

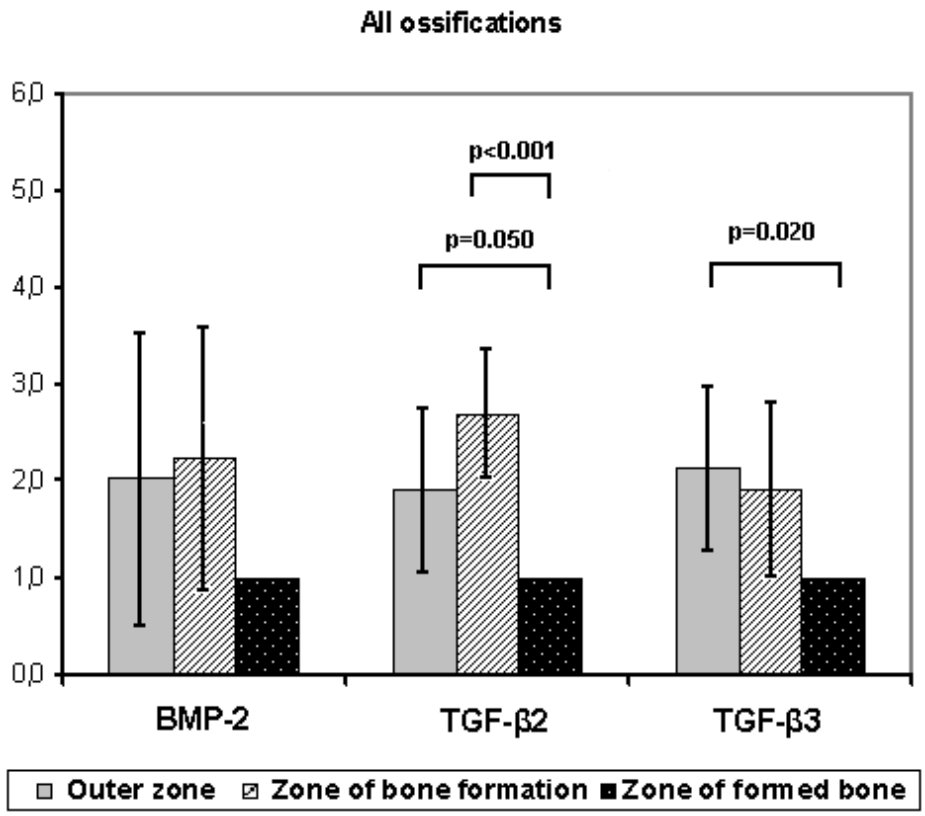

Figure 10. Expression of RNA for BMP-2, TGF-β_2 and TGF-β_3 genes in the different zones of all (n=7) ossifications normalised to the zone of formed bone from the same patient.

Ackerman was the first to report in 1958 morphological findings of HOs, that were formed after either accidental trauma or metabolic disturbances of tissues. These HOs developed after formation of the hematoma and necrosis followed by osteogenesis on the basis of organized tissues. He described, that HOs consist of a single ossicle with a specific structure [Ackermann 1958]. Later, Bosse [1997] described HOs in pressure sores that consisted of multiple ossicles. It should be pointed out, that in pressure sores there hardly exist any haematoma comparable by its extent to those appearing in surgical wounds and that the HO induction is related rather to the metabolic changes of the local environment. Disturbances of blood and oxygen supply as well as disturbances of innervation should also be considered [Bosse 1997]. In our samples of immature HO, we saw bone formation histologically similar [V] to the HOs of pressure sores [Ackermann 1958, Bosse 1997]. Bone was often formed as multiple small ossicles, which finally formed a conglomerate. These ossicles

grew intensively until they achieved maturity, after which slow remodeling and growth of bony part of ossicles continued. A similar principle of formation has also been described by other authors [Bosse 1997]. Hypothetically, such a multilocular appearance of bone induction may in early stages be caused by disturbances of osteoblast precursor proliferation and differentiation. A similar suggestion has been made also by later authors [Handschin *et al* 2006]. In general, it is accepted that HO after hip arthroplasty is a multifactorial phenomenon [Eulert *et al* 1997, Nilsson & Persson 1999, Pape *et al* 2004, Vanden Bossche & Vanderstraeten 2005]. One factor of HO induction that has been pointed out is the inexperience of surgeons [Nilsson & Persson 1999]. It could be hypothesized to be associated with more severe tissue trauma and thus more pronounced disturbances of the blood supply and innervation. More severe trauma also causes more active reaction of the organism to remove necrotized tissues that may lead to the higher concentration of prostaglandins at the operation site [Morykwas *et al* 1993], but prostaglandins are known as strong inductors of osteoblastic activity [Simon *et al* 2002, Ozturk *et al* 2005].

It has been found that in some diseases, such as fibrodysplasia ossificans progressiva, there exists an upregulation of the gene encoding BMP-4 [Vanden Bossche & Vanderstraeten 2005]. This mechanism of HO induction is supported also by experimental studies, which show that, using the BMP-4 inhibitor noggin, bone formation can be inhibited [Hannallah *et al* 2004]. In ossification of the yellow ligaments, BMP-2, -4, and -7 and their receptors have been found colocalized in the zone of ossification [Hayashi *et al* 1997, Tanaka *et al* 2001, Hayashi *et al* 1997]. Our findings regarding the BMP-2 protein and mRNA expression in bone formation zones are in concordance with their data, but further investigation of BMP-4 expression in HOs after THA may show even more prominent changes. Other risk factors for HO, possibly determined on the genome level, are male gender and hypertrophic type of osteoarthritis [I].

There are several studies based on the culturing of cells originating from HOs [Bidner *et al* 1990, Kaysinger *et al* 1997, Kurer *et al* 1992, Renfree *et al* 1994, Sell *et al* 1998] and one study where HO cells were harvested directly from the part of formed bone of patients with head injuries [Chauveau *et al* 2004]. Surrounding parts of the ossifications were, however, not included. Our method of microsurgical hard tissue preparation allows separation of different parts of the HO. Thus, it makes it possible to get information about biochemical and expressional changes that occur in adjacent areas. This helps understand the temporospatial changes occurring within the tissue, and our study [V] shows occurrence of both temporal and spatial changes within the HO.

Earlier information in literature almost always deals with immature HOs. In a study of Kaysinger *et al* [1997] the interval between the HO induction and sample harvesting was 9 months to 2 years; in a study of Sell *et al* [1998], it was 3–6 months; in a study of Chauveau *et al* [2004], it was 11 months on average [A. Toom, personal communication]; and in a study of Handschin *et al*

[2006] less than 2 years. Considering that bone remodeling is a slow process in adults that takes years to complete a turnover of bone mass, our study of mature HOs may help understand bone activity in persistent ossifications. There is one study considering HOs whose age from induction is not known. Puzas *et al* [1987], using supravital staining with tetracycline, reported that the linear apposition rate in HO was at least three times the normal rate of age-matched bone. Also, the counts of osteoclasts and osteoblasts were found to be higher in HOs.

It has been shown *in vitro* that osteoblasts originating from HOs have increased activities including synthesis of type I collagen that may be three times as high as in normal bone [Kaysinger *et al* 1997, Sell *et al* 1998]. Similarly, high bone turnover and rapid bone formation have been documented in HO tissues [Puzas *et al* 1989]. Further studies are needed to clarify the importance of collagen turnover in HO.

To sum up, high functional activity of bone cells as detected in HOs by earlier authors [Kaysinger *et al* 1997, Sell *et al* 1998, Chauveau *et al* 2004] as well as in our study [V] of immature ossifications indicates a higher remodeling activity during the first 2 years after HO induction.

Our study demonstrated higher bone formation activity of mature ossifications as reflected in the higher OS/Ps ratio compared to that for the control bone samples. Higher remodeling activity of mature ossifications was reflected in a tendency toward higher OS/Es than for the control samples, but this difference lacked statistical significance.

To our knowledge, our study [V] is the first comparing cellular activity and growth factor activity in mature and immature ossifications.

4.3. Specific effects of growth factors in bone formation zones of HOs

As visualized with immunohistochemical staining, TGF-β_1 protein seems to be universally present in many tissues (Figure 8A, B). This was our main consideration in using only quantification of TGF-β_2 and TGF-β_3 to determine possible fluctuations during bone formation. It is also well known that all three mammalian isoforms are synthesized and incorporated into the extracellular matrix during formation. Moreover, there are suggestions that action of TGF-β_2 is restricted to its site of formation [Janssens *et al* 2005].

Normalized levels of TGF-β_2 were increased in the bone formation zone of all HOs, while in the bone formation zones of immature HOs the expression of all studied growth factors higher than in the other studiedzones. The zone of formed bone in the youngest ossification consisted of woven bone, whereas in the remainder of the ossifications the bone tissue was mostly lamellar; therefore, normalized expression levels may not be directly comparable to the other ossifications.

TGF-β_1 and TGF-β_3 were predominantly expressed on the protein level by the differentiated osteoblasts, and their deposition in bone matrix was evident. TGF-β_2 was seen in immature ossifications and expressed mainly in preosteoblasts. This finding helps to clarify the function of this TGF-β subtype since an increase of both osteoblastic and osteoclastic activity, but with impaired matrix mineralization, has been reported for TGF-β_2 overexpression [Erlebacher & Derynck 1996].

Maeda and coworkers in 2004 found that signaling cross-talk between the BMP and TGF-β pathways plays a crucial role in the regulation of osteoblastic differentiation. Production of TGF-β is strongly induced during the maturation phase, as is production of inhibitory Smads (I-Smads). The authors supposed that this occurs through TGF-β mediation. TGF-β receptor kinase inhibitor then inhibits endogenous TGF-β signaling and suppresses expression of I-Smads. The decrease in I-Smads may result in acceleration of BMP signaling and enhancement of osteoblastic differentiation [Maeda *et al* 2004]. Similar cross-talk has also been proposed by other authors [Janssens *et al* 2005]. There is clear evidence that BMP-2 expression is higher during fracture repair [Liebermann *et al* 2002]. Sequential effects of these growth factors have been shown also during fracture healing in mice. BMP-2 peaked as early as 1 day after fracture, but TGF-β_2 and TGF-β_3 were strongly associated with extensive cartilage formation and collagen II production 7 days after fracture [Cho *et al* 2002]. In our study, the BMP-2 increment was barely significant since the range was wide. This is in accordance with previous data since we have occasional samples when BMP-2 was high and about to induce osteochondral development [Roark & Greer 1994, Cho *et al* 2002, Solheim 1998].

The higher expression of TGF-β_2 in the zones of bone formation in mature and immature ossifications and, even more, the higher expression of TGF-β_3 in the zone of cellular differentiation are similar to the data of Bosse and co-workers [Bosse *et al* 1994a]. Using digoxigenin-labeled cDNA probes, they demonstrated that TGF-β_1 was mostly expressed in locations where endo-chondral ossification occurred. This also coincides with our first report based on semiquantitative analysis of the RT-PCR product in ethidium bromide-stained gel electrophoresis, where we found more intense signal of TGF-β_3 in the surrounding zone and the weakest signal in the central zone with formed bone [Toom *et al* 2003]. Nevertheless, as detected by immunohistochemical staining, TGF-β_3 protein was synthesized in high concentrations in the walls of vessels, which are dense in the region surrounding the bone formation zone.

Summarizing this part of discussion, we were able to demonstrate [V], that there exists consequential manner of growth factor expression during the HO formation, and that the studied growth factors demonstrate co-operational properties.

5. Cellular sources of HO as studied in a rat model [VI]

After 3 days *in vivo* all implants had their pores mostly filled with a few inflammatory cells. There was no significant difference between the implants concerning the degree of cellular penetration. Some colonies of connective tissue cells with low differentiation were present, mostly in outer pores of implants. Their number was statistically different between the rhBMP-2 (group 2) and saline treated (group 1) implants: 5.22±1.67% and 1.31±0.35%, respectively, ($p=0.004$). The cellular content of the implants on the 3rd day did not reveal obvious difference between the intraoperative situations where femoral canal cells were present or absent. Histological investigation demonstrated in group 2 a high number of low differentiated connective tissue cells localized close to vessels in connective tissues surrounding the implants, whereas in group 1 such cellular activation was not evident (Figure 11). There was no difference between the groups 1A/2A and between 1B/2B.

After 21 days no bone tissue was formed in groups' 1A and 1B, except for one implant from group 1B where an osteoid region with a relative volume (BV/TV) of 0.35% was detected. In the other samples, osteoblast-like cells were only occasionally found on the surface of the implant, but without any recognizable osteoid or bone formation. Small amount of fibrous cartilage formation could be detected in the outer pores of all samples of groups' 1A and 1B, but there was no statistical difference between the groups. Nor was there any difference between groups 1A and 1B regarding the number of fibrous tissue cells, density of capillary sprouts and number of inflammatory cells. The implants were mostly surrounded by cell-rich fibrous connective tissue with fibers in arbitrary directions, which were observed similarly in both groups. There was no qualitatively distinguishable difference between the osteocalcin and osteonectin expression patterns as revealed by immunohistochemical staining, either.

Heterotopic bone was induced in all implants in groups 2A and 2B. Ossification occurred in the pores of implants and around the implants. All the implants were partly and some almost completely replaced by newly formed bone. Almost complete replacement of the implant occurred in one case of five in group 2A and in two cases of five in group 2B. A rigid bridging between the implant and the greater trochanter was seen in three cases of five in group 2A and in four cases of five in group 2B. There was no case of completely ossified bridging, instead, bridging consisted predominantly of calcified fibrocartilage or cartilage. The histomorphometric parameters of the formed bone are presented in the Table 7.

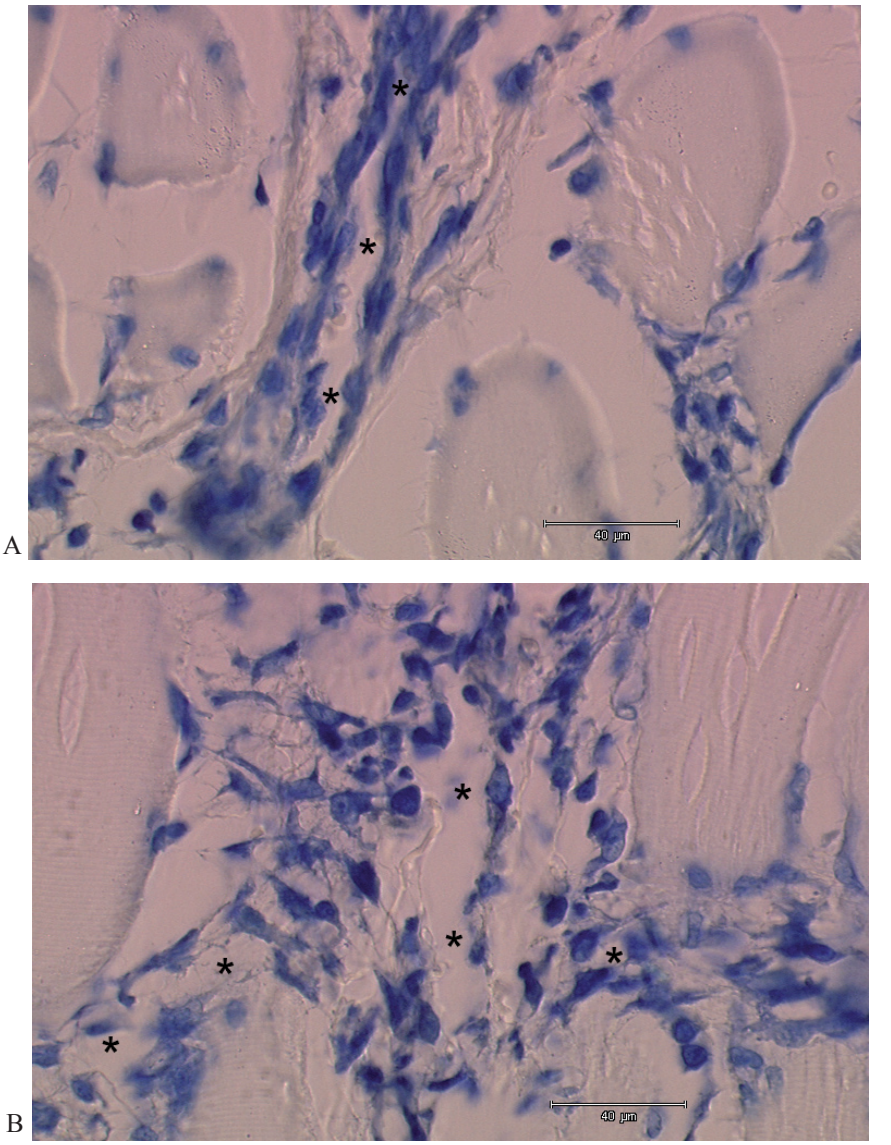

Figure 11. A. Perivascular cells are well orientated and close to the vascular walls in surrounding tissues of the implants in the group without osteoinduction. Vascular lumen (*). Staining with toluidine. B. Representative finding for the group with osteoinduction: perivascular cells are enlarged, their orientation in relation to the direction of vascular lumen is diverse and there is evidence of cellular translocation apart from the blood vessels. Vascular lumen (*). Staining with toluidine.

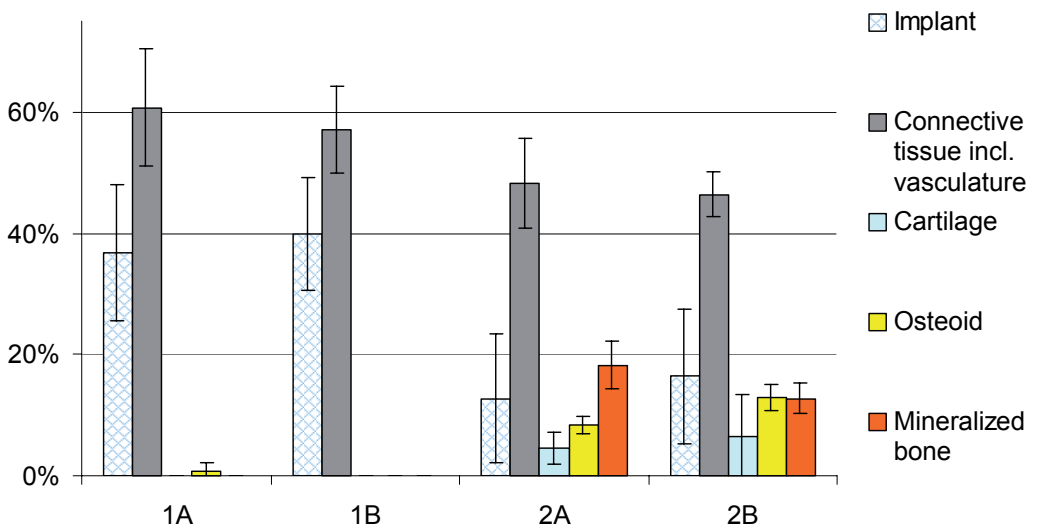

Figure 12. Volumes of different tissues and the implant after 21 days according to histomorphometric analysis.

Table 7. Characteristic histologic properties of the implants from different groups and relative volumes describing the properties of formed bone after 21 days of experiment.

Groups	1A	1B	2A	2B
3 days				
Cellular penetration into implant pores	+	+	++	++
Activation of perivascular cells				
21 days				
Bone formation	–	+/– [a]	++	++
Mineralization	–	–	+	++
21 days – relative volumes				
BV/TV	0%	>0% [a]	33.1% (SD 7.4%) [b]	30.0% (SD 7.6%) [b]
Md.V/TV	0%	0%	12.7% (SD 4.5%) [c]	18.2% (SD 2.9%) [c]
OV/BV	0%	100.0% [a]	57.3% (SD 5.0%) [d]	45.3% (SD 3.0%) [d]

[a] due to one case of osteoid formation
[b] paired t-test for groups 2A and 2B (p=0.234).
[c] paired t-test for groups 2A and 2B (p=0.019).
[d] paired t-test for groups 2A and 2B (p=0.010).

The ratio of osteoid surface to total bone surface (OS/BS), was 61.3% (SD 5.8%) in group 2A and 56.8% (SD 6.8%) in group 2B. There was no statistically significant difference between the groups (p=0.184). Similarily, the ratios of osteoblast surface to bone surface (Ob.S/BS) or the ratio of eroded surface to bone surface (ES/BS) did not differ significantly between the groups 2A and 2B.

Distribution of different tissue types is summarized in Figure 12.

Immunohistochemical study revealed no difference between the localizations of osteocalcin and osteonectin that could be distinguished by qualitative analysis. Staining for collagen I was used to increase the preciseness of the bone and osteoid volume estimations.

Our previous experience with establishment of HO in rats, using perforation of the femoral canal and leaving the debris created by drilling *in situ* in the capsulotomy wound [unpublished data], resulted in a very low rate of HO formation, even less than 50%. Therefore, we introduced a model of heterotopic ossification using exogenous implants. This model involved controlled bone induction by exogenous osteoinductive substance, which allowed elucidating the role of bone marrow stem cells in heterotopic ossification processes more precisely.

We used exogenous rhBMP-2 to standaridze the osteoinductive signal. Osteoinductive properties of exogenous BMP-2 have been well evidenced [Wozney *et al* 1988, Urist 1965].

One reason for choosing beta-tricalcium phosphate as the carrier for rhBMP-2 was its property to form a depot of recombinant BMP-2, as was shown by Uludag and co-authors [1999] as well as by other authors [Seeherman & Wozney 2005]. This avoids strong dilution and uncontrolled concentrations of rhBMP-2 in the implant region as well as its systemic effects. Moreover, it is well known that beta-tricalcium phosphate possesses excellent osteoconductive properties [Seeherman & Wozney 2005].

Many animal models of ectopic bone formation have been developed in order to investigate the mechanisms of HO formation, and its prevention as well as to assess the properties of different tissue engineering products. Mostly these authors make use of subcutaneous [Kantorowitz *et al* 1990, Jiang *et al* 2005, Kim *et al* 2005, Kroese-Deutman *et al* 2005, Liu *et al* 2005] or intramuscular pouches [Wang *et al* 1999, Vogelin *et al* 2000, Nakagawa *et al* 2003, Liang *et al* 2005] to achieve ectopic bone formation. Our method was intended to study heterotopic bone formation under standardized conditions (constant osteo-inductive signal, homogenous osteoconductive environment – beta-tricalcium phosphate, rhBMP-2). Another aim was to mimic better the situation after total hip replacement surgery. It is already long known from clinical practice that the HO is most common in the region of the abductor musculature, especially in the gluteus medius and gluteus minimus muscles [Kjaersgaard-Andersen *et al* 1990, Ahrengart 1991, Bisla *et al* 1976, Søballe *et al* 1988, Nollen & Slooff 1973, Puzas *et al* 1989]. This region corresponds to the structures subjected often to tensile forces and which is damaged during the total hip replacement surgery. The same localization of HO was used also in our model. We localized the implant close to the greater trochanter, immediately under the abductor musculature, and connected with the capsulotomy wound.

Heterotopic bone formation without exogenous bone induction

There was no significant bone formation either in the presence (group 1A) or in the absence (group 1B) of femoral canal cells during a relatively short time period (21 days) unless an additional osteoinductive factor was applied. Exceptionally, minimal osteoid formation was recorded in a limited localization in one subject from group 1B. Considering that in this case the femoral canal cells were absent, this kind of osteoinduction has to be applied to local factors and to the osteoprogenitor cells activated by local trauma.

Heterotopic bone formation with exogenous bone induction with rhBMP-2

3 days after induction large areas of implants were penetrated by the low differentiated connective tissue cells. Later, bone formation occurred on the outer surface of the implant. No polarity of ossification was detected in the implants, providing evidence of cellular migration from all directions into the implant. Absence of polarity favours the hypothesis that osteoprogenitor cells responsible for heterotopic bone formation in the proximal femoral region

originate from the surrounding soft tissues like the joint capsule, the blood vessels, the tendon- and the muscle sheets, and probably also from the periostal pool. Also, the histologic finding of "wandering" perivascular cells in surrounding tissues of implants 3 days after induction supports this hypothesis.

Osteoid volume (OV/BV) was very high in both groups, 2A and 2B (Table 7), which indicates a high rate of formation of new bone. Thus, the HO formation was evident in both hind limbs in all animals. It is difficult to explain the significantly lower mineralized volume of bone (Md.V/TV) on the right side, while the volume of formed bone (BV/TV) itself was similar on both sides (Table 7). This may be due to an increased washout of rhBMP-2 caused by bleeding from the femoral canal. Yet some inhibitory factors originating from the femoral canal cannot be ruled out. In fact, formation of complete bone structure was delayed in the group with osteoinductive signal and opened femoral canal (Group 2A in Table 7).

Our results are in concordance with a prospective randomized study in arthroplasty patients where pulsative irrigation was used to remove bone dust (containing osteogenic cells) from the femoral canal. In this study no difference was found between the treated and control groups [Sneath *et al* 2001]. Moreover, a clinical study showed that the gluteus minimus debridment had a positive effect through reducing the incidence of HO after acetabular fractures [Rath *et al* 2002]. However, this study involved no adequate control group. There are controversial results that are indicating the priority of femoral canal cells in rabbits [Rumi *et al* 2005b]. In their experiment either the femoral canal or surrounding muscules in the proximal femoral region are irradiated and the formation of HO is assessed by modified grading-score based on Brooker's score [Brooker *et al* 1973]. The discordance between the results of Rumi and coworkers [2005b] and ours [VI] might be difficult to explain. However, Rumi and co-workers used rabbits, an animal species with known predisposion to react to the forcible mobilization of muscles with HO formation [Schneider *et al* 1998]. On the other hand our results from the rat experiment [VI] are in concordance with the clinical findings in humans [Sneath *et al* 2001, Rath *et al* 2002, Nilsson & Persson 1999]. Thus it may be indicative of the fact that the rat model of HO stands closer to the clinical situation than the rabbit-model.

The rabbit model of heterotopic bone formation by Schneider and co-workers [1998], which mimics the real situation after hip replacement surgery, has a disadvantage regarding the variable degree of bone induction. The method of Kantorowitz and co-authors employs a non-physiological situation where HO is created in a subcutaneous localization [Kantorowitz *et al* 1990], which can be avoided with our method. By determining the exact degree of the osteoconductive and/or osteoinductive factors, our method allows to diminish the number of subjects included in assessment of different local factors.

One limitation of our study is fact, that in all subjects and in all groups a similar muscle injury was induced. A matched study applying our method of standardized HO induction [VI], where the extent of muscular damage is varied, would be a way to confirm the association between the HO formation and the priority of the extent of trauma in the development of HO.

GENERAL DISCUSSION

Formation of HO is a remarkable situation in the sense of tissue metaplasia. The new tissue has a complicated structure with spontaneous growth deceleration. We here evaluate its origins and inducing factors, how to diagnose HO and how growth factors act internally in the HO.

1. Recording of the incidence and predisposing factors

The incidence of HO in our clinic (32%; [I]) was somewhat lower than the incidence reported in the largest meta-analysis, 43% [Neal *et al* 2002]. This may appear high prevalence, but clinical manifestations are actually rather seldom present. When related to the grading system by Brooker scale [Brooker *et al* 1973], clinical symptoms were evident mainly in grades III and IV. Prevalence of serious cases when all grades are combined has been reported to be 9% [Neal *et al* 2002]. Data from the clinic where this study was performed had a lower incidence, being 4% [I]. Physicians cannot only depend on the grading [Brooker *et al* 1973], since our results showed that sometimes also the grade I may involve extensive ossification [II, III].

We have found an increased incidence of HO in association with the following factors: male gender, THA of the contralateral hip, previous surgery to the hip, lack of preoperative treatment with NSAIDs and the duration of operation more than 100 min. These findings in general are consistent with those of other authors. Differences in HO incidence are probably to be found among these factors. Many authors have reported a higher incidence of HO in males [Ahrengart 1991, Ahrengart & Lindgren 1993, DeLee *et al* 1976, Duck & Mylod 1992, Hirota *et al* 1997, Pedersen *et al* 1989, Shaffer 1989], which was confirmed in this study. The influence of age on the development of HO is controversial. A higher incidence has been reported in elderly women [Ahrengart & Lindgren 1993]. Other authors, on the contrary, have reported no correlation with age [Pedersen *et al* 1989], as is confirmed by our study, although our HO patients were a little older (not statistically significant) [I]. There are reports of an increased incidence of HO in association with extensive osteophytosis and with the hypertrophic type of osteoarthritis [Ahrengart 1991, Ahrengart & Lindgren 1993, Goel & Sharp 1991]. This was, however, not evident in our study [I], nor in the report by Pedersen *et al* [1989]. Many authors have shown that pre-existing HO of contralateral hip is associated with an increased incidence [Ahrengart 1991, Duck & Mylod 1992, Hirota *et al* 1997, Kilgus *et al* 1990, Pedersen *et al* 1989, Sodemann *et al* 1988, Warren 1990]. DeLee *et al*. [1976] report an incidence of 92% in patients who developed HO after contralateral THA. Grade IV HO occurred in seven cases in this study, of which four

had developed grade II or grade III ossification 6 months after THA, and had developed grade IV ossification after contralateral THA. The presence of significant differences in distribution among the patients who had previously undergone surgery on the contralateral hip – and in fact there was no difference in distribution among the patients who had previously undergone surgery to the ipsilateral hip – may be related to the nature of the procedures. The contralateral operations were mainly arthoplasties (15 cases out of 19), whereas none of the previous ipsilateral procedures was a THA. A similar pattern of HO was demonstrated by Sodemann and co-authors [1988], but not, however, by Vastel *et al*. [1998].

Many reports have shown causal relationship between tissue trauma and the formation of HO [Hierton *et al* 1983], probably related to local vascular disturbance and necrosis [Ahrengart *et al* 1987, Hierton 1983, Shaffer 1989]. The effect of these factors was increased with longer operating time, which has also been shown to be associated with a higher incidence of HO by other authors [Soballe *et al* 1988, Sodemann 1988]. There are, however, other reports showing no relationship between the incidence of HO and the length of operation [Pagnani *et al* 1991, Vastel *et al* 1998]. This factor therefore may not be too important for appearance of HO.

We have also observed a lower incidence of HO in patients of the 0 blood group [I]. To our knowledge, there are no confirming reports. The association between the development of HO and the AB0-antigen system indicates that HO might be related to genetically predetermined factors. There are some reports on the connection between the specific alleles of HLA-system and neurologic HO, but these data cannot exclusively confirm any immutable predictor for HO [Garland *et al* 1984, Larson *et al* 1981, Van Kuijk *et al* 2002, Weiss *et al* 1979]. However, presence of such repeated reports is indicative of genetic pre-determination.

Summing up, the incidence and severity of HO can systematically vary between clinics. Further studies are needed to sort out the crucial factors for an adequate selection of patients for prophylactic treatment. We believe this can be achieved in prospective studies.

2. Improvement of the classification system

As the analysis of digitalized planimetry demonstrated, the main source of error in assessment of severity of HO was the error of recognizing the HO [IV]. Similar studies have been performed also with other x-ray assessment systems. It has been shown, that there was no significant influence of the assessment method, if digital or conventional x-ray techniques were used to diagnose either configuration of hip joint [Essmann *et al* 2006], severity of aseptic loosening in

after THA [Eklund *et al* 2004] or applicaton of Larsen classification of rheumatoid arthritis [Young-Min *et al* 2003].

We agree completely with Eklund and co-authors who pointed out also the need for higher qualification of examiners [2004], but it should be emphasized that reliability in practical clinical use would be higher if the assessment system consists of clearly understandable criteria, which need fewer efforts to learn and apply them properly.

Computer aided methods can improve, but their usage is still limited in this respect [Eklund *et al* 2004, Essmann *et al* 2006, Young-Min *et al* 2003, IV]. Generally physicians need classification systems that excert higher reproducibility, are easier to learn and introduce and have a higher accuracy.

Our results demonstrate that the incidence of HO varies considerably depending on the rating system used (Table 3). This fact has to be considered if studies of different authors are compared. Our data demonstrate that the most widely used, the Brooker's classification does result in lower inter-observer reliability than other systems assessed. The other classifications had very similar values of the kappa statistic.

Combining Brooker's [Brooker *et al* 1973] classification with Della Valle's [Della Valle *et al* 2002] system we were able to provide adequate inter-observer reproducibility and to improve consistency in classifications of HO. Despite the fact that Della Valle's system is basically not comparable to previous studies because of its different criteria, our combined classification is easily convertible to both of its parent systems. Reproducibility of this part of the classification was basically same as in Della Valle's system. Our assessment gave a satisfying kappa value. We hope more comparisons will be made, since it is probably a useful way forward.

3. Towards understanding of the pathogenesis of HO

There are three conditions, as supposed already in 1975 by Chalmers and co-workers, that unavoidably should be fulfilled to initiate the development of HO – presence of osteoprogenitor cells, osteoconductive environment and osteoinductive factors. The first two conditions are always fulfilled in muscular tissue and fascias, due to the nature of the tissues. Osteoinduction should, on one hand, be considered a primary condition like in genetic disorders or after trauma or bone sawing, where liberated deposits of BMP's and TGF's act as inducers of ossification. In case of trauma such osteoinduction belongs to the physiological adaptive reaction. In case of heterotopic ossification this reaction occurs where it is not necessary. Here a combination of other conditions play a role. For example, increase in the pro-inflammatory prostaglandins may be responsible for creating such a condition as shown in animal experiments [Simon *et al* 2002, Suutre 2004]. However, in humans its relevance is still under discussion

[Einhorn 2002, Aspenberg 2005]. As vascular injury and disturbances of innervation are present in all situations (except conditions related to gene over-expression) where HO occurs. They would be set as the next level in this cascade. Finally, the cascade will continue under the influence of systemic factors, that should not always be present, and with predisposing genetical determinants, that are currently most disputable. In summary, we draw up a putative scheme (Figure 13).

Of course, there remains the possibility that some other conditions may exert influence too because it is known that osteoblasts in HO have continuously higher activity [Puzas *et al* 1987] and this is lasting even many years after HO induction [V]. We can propose that there may be factors directly affecting the auto- or paracrine regulation of osteoblasts, as the increased expression of growth factors was demonstrated in the borderline of HO even 9 years after induction [V]. Factors that activate the osteoblasts do not necessarily have to originate from the HO itself, also systemic factors or their release them from adjacent tissues may be involved.

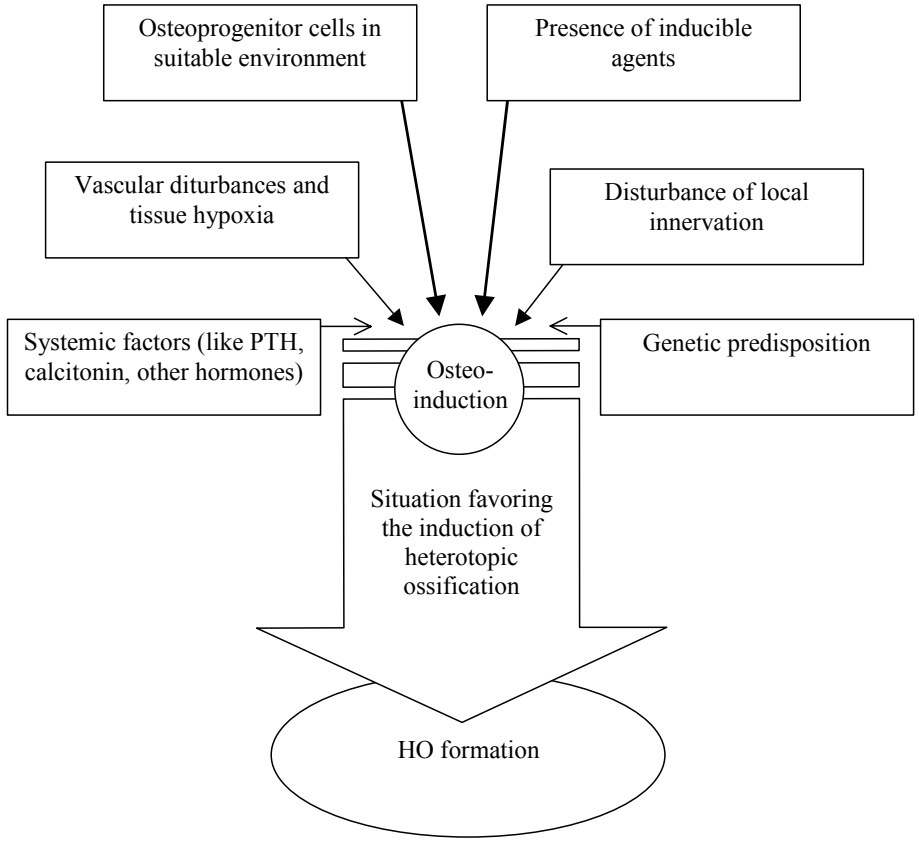

Figure 13. Schematic presentation of pathogenic factors required for HO induction.

There are several studies where the purpose of the investigations has been assessment of the risk factors for HO. Risk factors has been found in association with hypertrophic osteoarthritis, contralateral total hip replacement, previous hip surgery, and subtrochanteric femoral osteotomy, surgical lateral or anterolateral approach, very high body mass index, repeated surgery and male gender [Vastel *et al* 1998, Nilsson & Persson 1999, Eggli & Woo 2001, I]. Actually, also traumatizing of surrounding muscles [Hierton *et al* 1983, Shaffer 1989, Puzas *et al* 1989, Nilsson & Persson 1999], as well as extended exposure to mechanical factors in the conditions of long operative times have been shown to be risk factors for HO [I]. The above data support the idea that local damage induces HO.

In our animal experiment the surrounding muscular tissues and the joint capsule were injured in a similar way, while we found no differences in the intensity of bone formation between the groups in which femoral canal cells were either present or absent [VI]. Faster calcification of formed bone detected in the group with the absence of the femoral canal cells, may be a somewhat confusing fact, but this experiment indicates, that the main operation related cause of heterotopic ossification is creation of a suitable environment and increasing the inducing signal in surrounding tissues. This is in accordance also with the findings of authors who have shown positive relationship between HO and severe tissue trauma [Nilsson & Persson 1999] or longer operating times [Eggli & Woo 2001, I]. In the discussion of cellular sources of HO one report demonstrating the inductive ability of circulating monocytar lineage cells has to be mentioned, too [Buring 1975].

The unavoidable conditions for HO, such as presence of osteogenic, osteoinductive and osteoconductive factors, can in general be found in the surgical wound in case of THA. If the operative trauma is more severe, larger tissue damage is inflicted that leads to the more prominent disturbances of the blood supply and the local innervation.

Once the suitable conditions are created the cascade of osteogenic differentiation and bone formation is activated.

The retainment of high osteoblastic activity over years as shown by many authors [Kaysinger *et al* 1997, Puzas *et al* 1987, Sell *et al* 1998, V] is confusing. As we were able to demonstrate, this condition is maintained even after 9 years [V]. One hypothesis is that it is related or dependent on organism related predisposing conditions. This hypothesis is supported by the findings about the association of HO with blood group systems [I] and the HLA [Garland *et al* 1984, Larson *et al* 1981, Van Kuijk *et al* 2002, Weiss *et al* 1979]. The existence of growth factor overexpression syndromes that lead to HO formation [Hannallah *et al* 2004], and even the widely known fact that male gender is at higher risk for HO are other possible factors.

Another hypothesis might be based on the similarity between the borderline of matured HOs and the tendon enthesis [V]. It is known that the calcified

fibrocartilage zone of tendon enthesis is functioning as a secondary growth plate [Benjamin *et al* 2002] similarly to the calcified articular cartilage zone [Oegema *et al* 1997], being a region of active bone turnover. Further studies are needed to clarify the continued presence of osteoblastic activity.

As we normally cannot influence the genetically related conditions, a better principle would be to evaluate clinical management in the operating theatre. We can reduce trauma by operating more carefully, while prophylactic agents should be considered only in patients with conditions predisposing for HO.

CONCLUSIONS

1. The incidence of HO after after total hip arthroplasty in our clinic was 32%; the rate of severe cases was 4%. We have found an increased incidence of HO in association with the following factors: male gender, THA of the contralateral hip, previous surgery to the hip, lack of preoperative treatment with NSAIDs and the length of operation of more than 100 min. A lower incidence of HO was observed among patients of the 0 blood group.

2. Our analysis revealed that the incidence of HO after THA is dependent to a large extent, on the classification system used. We propose a combined classification system providing easier clinical assessment and higher inter-observer reliability. The classification is convertible to previous studies using Brooker's and Delle Valle's classifications.

3. The main source of error in diagnosing HO using digitalized planimetry occurs during estimation of HO on radiographs. Technical error of image processing by a computer program influenced total error less and the subspecialization of doctors did not cause systematic bias.

4. A microscopy-guided tissue separation method was applied in order to compare gene expression in adjacent tissues. Histomorphometric analysis revealed high osteoblastic activity reflected as an increased ratio of osteoid surface in HOs. Additionally, we found that TGF-β_2 expression and TGF-β_3 expression are increased during bone formation as well as during bone remodeling. We found that mature HOs showed slightly higher OS/Ps ratios in comparison to control bone, indicating that high remodeling activity is maintained in old HOs. Our results suggest that HO has high bone-forming activity, with the growth factors BMP-2, TGF-β_1, TGF-β_2, and TGF-β_3 involved in this process, and that as time advances bone-forming activity slows down.

5. Presence of femoral canal cells does not seem to exert an additional osteoinductive or osteogenic effect. Based on the data of current experiment, it can be suggested that in the presence of a strong osteoinductive signal (local rhBMP-2 treatment), the cells or the substances originating from the femoral canal might be even inhibitory to mineralization processes in newly formed bone.

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SUMMARY IN ESTONIAN

Heterotoopne ossifikatsioon puusaliigese totaalendoproteesimise järgselt: kliiniline ja patogeneetiline uurimus

Heterotoopse ossifikatsiooni (HO) all mõistetakse luustumisprotsessi, mis leiab aset väljaspool skeletisüsteemi. HO ei ole lihtsalt kaltsifikaat, mis tekib kaltsiumisoolade ladestumisel kudedesse. Tõelisest HO diagnoosist saame rääkida vaid juhul, kui on moodustunud luukude koos luurakkude, iseloomuliku kollageenmaatriksi ja hüdroksüapatiidi kristallidega. HO ei ole kasvajalise iseloomuga, tekkinud luukoes ei esine rakulist ega koelist atüpismi.

Heterotoopset ossifikatsiooni esineb puusaliigese totaalendoproteesimise (TEP) järgselt väga sageli. 2002 aastal teostas Neal koos kaasautoritega seni-ajani kõige ulatuslikuma meta-analüüsi, mis hõlmas 59 121 patsienti. Selle töö andmetel esineb puusaliigese TEP järgselt HO (kõikides raskuastmetes kokku) 43% endoproteesitud patsientidel [Neal *et al* 2002].

HO esinemissageduse ulatuslikku varieeruvust erinevates uuringutes võib seletada heterogeense patsientuuri, varieeruvate operatsiooni- ja järelravi-meetoditega ning olulisel määral ka hindamiskriteeriumide- ja meetodite erinevustega. Teatud mõju on ka kliinilisel kogemusel [Nilsson & Persson 1999].

HO esinemissagedus on küll kõrge, kuid kliinilisi kaebuseid põhjustab see patoloogia suhteliselt harva. Suuremal osal juhtudest on lihtsalt tegemist asümptomaatilise röntgenoloogilise leiuga. Kliinilist sümptomaatikat esineb tavaliselt raskemate HO juhtudega, mis klassifitseeruvad Brookeri skaala järgi III ja IV klassi [Brooker *et al* 1973]. Selliste raskete juhtude esinemissagedus oli erinevate autorite hinnangute summeerimisel meta-analüüsis 9% [Neal *et al* 2002].

Uurimuse eesmärgid

Määrata puusaliigese totaalendoproteesimise järgse HO esinemissagedus ja selle raskusastmed ning selgitada HO tekkeks predisponeerivad tegurid [originaalpublikatsioon I].

Võrrelda erinevate klassifikatsioonisüsteemide mõju HO esinemissagedusele ja pakkuda välja kõrgema usaldusväärsusega süsteem [originaalpublikatsioonid II ja III].

Leida HO röntgenoloogilisel hindamisel tekkiva vea allikad, arvutada erinevate veakomponentide suurus ning teha kindlaks, kas arvutiprogrammi rakendamine hindamise abistamiseks aitab olulisel määral parandada hindamise täpsust [originaalpublikatsioon IV].

Kirjeldada HO morfoloogiat dünaamikas ning hinnata osteoinduktiivsete kasvufaktorite (BMP-2, TGF-β_2 ja TGF-β_3) ekspresseerumist heterotoopse luu moodustumise käigus [originaalpublikatsioon V].

Modelleerida HO moodustumist roti mudelis eesmärgiga uurida osteoprogenitoorsete rakkude päritolu [originaalpublikatsioon VI].

Materjal ja meetodid

I artikkel käsitles 178 patsienti, kelle puhul selgitati retrospektiivselt HO esinemissagedus Tartu Ülikooli Kliinikumis totaalendoproteesimise järgselt ning selgitati peamised predisponeerivad tegurid. II ja III artikkel analüüsisid 111 patsiendi röntgenülesvõtetetele tuginedes erinevate klassifikatsioonide kasutamise mõju HO esinemissagedusele ja uurijate antud hinnangute kooskõla; III artiklis koostasime enimlevinud HO klassifikatsiooniga ühilduva kõrgema usaldusväärsusega süsteemi. IV artiklis rakendasime 28 patsiendi digitaliseeritud röntgenülesvõtetele antud hinnanguile dispersioonmudelit, leidmaks HO diagnoosimisel tekkiva hindamisvea allikaid. V artiklis kirjeldasime 19 patsiendilt ja kontrollisikult kogutud operatsioonimaterjali põhjal HO morfoloogiat ning uurisime luu histomorfomeetrilist analüüsi, semikvantitatiivset pöördtranskriptaasi reaktsiooni ja immuunhistokeemilist uuringut kasutades luurakkude aktiivsust ning luu kasvufaktorite osalust ossifikaadi arengu käigus. VI artiklis modelleerisime 20 katseloomal totaalendoproteesimise operatsiooni ning uurisime heterotoopse luu moodustumist rakendades luu histomorfomeetrilist analüüsi ja immuunhistokeemilisi uuringuid.

Olulisemad tulemused ja järeldused

Puusaliigese totaalendoproteesimise järgse HO esinemissageduseks oli meie kliinikus 32%; sh raskete juhtude sagedus 4%. Leidsime, et HO esinemissagedus oli suurem järgmiste riskitegurite olemasolul: meessugu, totaalendoproteesimine kontralateraalsel liigesel, varasem puusaliigese operatsioon, preoperatiivse mittesteroidsete põletikuvastaste preparaatide kasutamise puudumine ning operatsiooni kestus üle 100 minuti. HO esinemissagedus oli madalam AB0-süsteemi 0-vererühmaga patsientidel.

Meie analüüs näitas, et HO hinnang esinemissagedusele sõltub oluliselt rakendatavast klassifikatsioonisüsteemist. Pakkusime välja kombineeritud klassifikatsioonisüsteemi, mis võimaldab mugavat rakendamist kliinilises praktikas ning säilitab kõrge hindamistevahelise usaldusväärsuse. Selle klassifikatsiooni kriteeriumide kasutamisel saadud tulemused on täielikult võrreldavad Brooker'i ja Delle Valle süsteemide kriteeeriumide kasutamisel saadud tulemustega.

Olulisima kaaluga veaallikaks HO diagnoosimisel digitaalplanimeetrilisel meetodil oli röntgenogrammide hindamisel tekkiv viga. Arvutiprogrammi kasutamisega välditav tehniline viga oli statistiliselt oluline, kuid väiksema kaaluga. Arstide ettevalmistuse eripärad ei põhjustanud süstemaatilisi kõrvalekaldeid.

HO uurimiseks eesmärgiga hinnata geenikspressiooni lähedalasuvates kudedes rakendati mikroskoobi kontrolli all teostatavat kudede separatsioonimeetodit. Histomorfomeetrilisel analüüsil selgus, et HO-s on tegemist kõrge osteoblastilise aktiivsusega, millele vastas kõrgenenud osteoidi pinna suhtarvu tõus. Lisaks sellele leidsime, et nii heterotoopse luu moodustumise kui ka selle remodelleerumise käigus esineb TGF-β_2 ja TGF-β_3 ekspressiooni suurenemine nii mRNA kui valgu tasemel. Leidsime, et lõplikult formeerunud HO puhul esines normaalse luuga võrreldes mõningane osteoidi pinna/periostaalse pinna suhte kõrgenemine, mis viitab remodelleerumise kõrgenenud aktiivsuse säilimisele vanades HO-des. Meie tulemused osundavad, et HO-s on luumoodustumise aktiivsus kõrgenenud ning selle säilitamisse on kaasatud kasvufaktorid BMP-2, TGF-β_1, TGF-β_2 ja TGF-β_3.

Reieluu üdikanali rakud ei avaldanud HO formeerumisele ei osteoinduktiivset ega osteogeenset lisamõju. Tugeva osteoinduktiivse teguri olemasolul (rhBMP-2 lokaalne manustamine) võiks käesoleva katse alusel oletada üdiõõne kanalist pärinevatel substantsidel või rakkudel isegi moodustunud luu mineraliseerumist pärssivat toimet.

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PUBLICATIONS

I

Toom A, Haviko T, Rips L.
Heterotopic ossification after total hip arthroplasty.
Int Orthop, 2001; 24(6) 323–6

II

Toom A. **Heterotoopse luustumise hindamine: erinevate klassifikatsioonide võrdlus [Assessment of heterotopic ossification: comparison of different classifications***; article in Estonian***]**. *Eesti Arst*, 2003; Lisa 6: 7–11

III

Toom A, Fischer K, Märtson A, Rips L, Haviko T.
Interobserver reliability in the assessment of heterotopic ossification: proposal of a combined classification. *Int Orthop,* 2005;29(3):156–9

IV

Toom A, Möls M, Fischer K, Uibo R, Veske K, Selstam G, Haviko T, Arend A, Märtson A. **Digital planimetry for determination the severity of heterotopic ossification and sources of assessment errors** (manuscript)

DIGITAL PLANIMETRY FOR DETERMINATION THE SEVERITY OF HETEROTOPIC OSSIFICATION AND SOURCES OF ASSESSMENT ERRORS

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ABSTRACT

Heterotopic ossification (HO) is a frequent pathological phenomenon after total hip arthroplasty. Incidence of HO after the total hip arthroplasty (by meta-analysis) is in average 43%.

The aim of the present study was to determine the sources of errors in the assessment-process of HO and, to reveal in which degree computer assistance can improve the reliability of assessment.

Six investigators measured dimensionality of HO in x-rays of 28 patients applying method of digital planimetry. Main sources of errors were detected by dispersion model.

The most important source of error in the HO assessment was the diagnosing process. This source consisted of two components: inter-observer variation that formed 25.5% (±8.0%; $p=0.0015$) of total error and intra-observer variation that formed 60.9% (±7.3%; $p<0.0001$). Technical performing error had less contribution in total error, namely 8.0% (±0.6%; $p<0.0001$) and subspecialiation of the investigators did not cause any systematic bias having a proportion of 5.7% (±4.9%; $p=0.2457$).

INTRODUCTION

Heterotopic ossification (HO) is an ossification process occurring outside of skeletal system. HO is not simply calcification, when calcium salts are deposited into the tissues. True HO is formed only in case when bone tissue is

present, which contains bone cells, characteristic collagen matrix and hydroxoapatite crystals is appeared (Puzas *et al* 1989).

HO is very common after total hip arthroplasty (THA). The most recent and wide meta-analysis, which took into consideration data of 59121 patients was performed by Neal *et al* in 2002. By this work HO (all scores) appeared in 43% of patients who underwent total hip replacement (Neal *et al* 2002). By the retrospective study performed in Tartu University Hospital on 178 patients operated in 1995–1996 the prevalence of HO was 32%, including 4% of serious cases (Toom *et al* 2001).

Diagnosis of HO is based on the radiological findings. Currently the most widely used classification for assessment of the HO is Brooker's system proposed in 1973 (Brooker et al 1973). Still, the reliability of the system is not the best. The interobserver reliability based on Cohen's kappa-value has reported to be "fair" 0.50 (95%CI 0.30–0.70) (Neal et al 2002). Same problem has been faced also earlier – in 1994 Brooker's classification was revised and an interobserver kappa rose from 0.57 to 0.68 if additional criteria were applied (Wright et al 1994). We have been able to demonstrate that the incidence of HO can vary considerably (even 1.4 times) depending on the rating system used (Toom et al 2005). To increase the reliability we recently proposed a new classification method (Toom et al 2005), which combined system of Brooker et al with the system of Della Valle and co-authors (2002).

These data clearly suggest the need for improvement of the classification system and therefore the understanding of possible source of errors of the assessment is essential.

Of course, the best control of roentgenological estimations is to compare it with histological and histomorphometrical investigations. The ossificates can be obtained only in re-operations, but as these operations are rare this methodology is difficult to use.

Planimetry method has been widely used in determination of wound areas (Langemo et al 1998), in histomorphometric analysis (Hussar et al 2001, Masso et al 2003) and also in computerized tomography (Sell et al 2003). A quantitative method based on digital planimetry has been proposed for assessment of HO (Schoellner *et al* 2000). Shoellner's system divides ossifications into ten groups independent of their localization. The minimal size of ossification what is possible to detect by the use of digital planimetry is 0.1 cm^2 (Schoellner *et al* 2000).

The method is technically difficult and time consuming, which makes it not suitable for everyday use. Digital planimetry estimation contains methodically two stages – diagnostics of image and technical subscription of marking the image, which gives as a result mathematically estimated nominal values. We decided to use this method to estimate a source of errors in classification of HO.

The aims of the study were to calculate value and estimate sources of errors appearing in the assessment of HO on plain x-rays and, based on the data to

appraise, whether application of computer-assisted measurements of HO can significantly improve the HO assessment.

MATERIALS AND METHODS

28 patients to whom total hip replacement was performed in 2002 in Department of Orthopaedic surgery of Tartu University Hospital were recruited into the study. These patients were randomly selected from those who were enrolled into a larger heterotopic ossification study in the same hospital (Estonian Science Foundation grant 5210) after they had roentgenological documentation of the presence of HO. The group consisted of 15 female and 13 male patients with the average age of 69.9 (range 49 to 83) years and 61.6 (range 32 to 78) years, respectively. All patients received 200 mg celecoxib (Celebrex, Pfizer, USA) or 100 mg diclofenac-sodium once a day during a one-week course. Antithrombosis prophylaxis was performed with low-molecular weight heparin at least 5 days.

Visual assessment system
Three observers assessed the x-rays using our classification system (Toom et al 2005) at three different time-points: two of them were consultant-surgeons and one of them a trainee of orthopaedic surgery. The analysis was performed blind regarding the other analyses. All of them were previously familiar to that system. They all were asked to have a printed table of this system always available. Observers were restricted to discuss the criteria and cases during analysis-period.

Digital planimetry
Frontal plane (plain) x-rays were used for assessment. Anterioposterior x-rays were taken immediately after operation in recovery room and 12 to 14 months later during the follow-up. All the pictures were digitized and all the assessments were performed using the same views of the pictures. Adobe Photoshop version 5.0.2 was used for the estimation of the extent of HO as described earlier (Hussar et al 2001). The earlier relative method was supplemented in this work by the calibration that allows to make absolute estimations about the size of HOs. The calibration of x-rays used known isotropically projected (bilaterally symmetric) measures of total hip prosthesis (neck diameter of femoral component and femoral head edge measure). Each observer made calibration performing triple measurements of the two constant measures of the endoprosthetic head applying the “measure tool” (Figure 1). The result of calibration was the known area of one pixel.

Thereafter ossifications were delineated using the “polygonal lasso tool” (Figure 2A), and marked by separate colour (Figure 2B) not presented on the

gray-scale digitized pictures. All coloured areas were measured using "histogram" function (Figure 2C). Procedure was repeated three times.

Similar measurement procedure, excluding the picture calibration, was repeated by each observer 12 weeks later.

After entering the numerical measurements into the computer-based spreadsheet, the areas of ossifications for each measurement were automatically calculated and converted into the classification system proposed earlier (Schoellner et al 2000).

To minimize qualification bounded errors together with technical ones six investigators were included. Two of them were orthopaedic surgeons, two radiologists and two morphologists. Estimation was performed in two sessions with an interval of 3 months. During the session observer detected the ossifications and marked them up by the use of computer program repeatedly three times. This gave possibility to calculate technical errors and diagnostic errors between each observer. Two different sessions gave possibility to calculate intraobserver error of the diagnosing. Estimation took place as a blinded study and observers were not allowed to discuss about results or share opinions.

Statistical analysis.
Dispersion model was made by use of computer program STATISTICA. Statistical significance was defined as $p<0.05$.

RESULTS AND DISCUSSION

Results of digital planimetry assessment are described by formula:

$$Y_{ijkl} = a_i + b_j + c_{ij} + d_{ijk} + e_{ijkl};\ \text{where}$$

(Y) is measured result; (i) patient number; (j) examiners number; (k) number of session and (l) number of repeated estimation; (a), (b), (c), (d), and (e) – different functions to different arguments. So the dispersion components have the following meaning:

- a_i variation coming directly from the x-ray, calculated as the total average of all examiners and all observations;
- b_j systematic variation of every single examiner (biased error)
- c_{ij} variation arising from examiner and x-ray co-action – describing variation between examiners
- d_{ijk} variation arising as co-action of every examiner, x-ray and observed session – describing variation of examiner
- e_{ijkl} variation arising from the detecting and selecting image by every examiner so-called technical error or methodological error

The estimation of dispersion component corresponding to every parameter is given in Figure 3 and the statistical significances in the Table 1.

Figure 3 and Table 1 demonstrate that the main part of the measured values is coming from x-ray itself and dispersion arising from the different observations is considerably smaller. The largest part of dispersion of digital planimetry arose from the x-ray itself. This indicates clearly that the method of digital planimetry shows the differences that have been found objectively on the x-ray images and thus, really enables to estimate the measures of HO on the x-rays. More important sources of dispersion are differences between the estimations of different examiners as well as between the different diagnoses/observations of the same examiner.

Whilst one of possible sources of errors could be the qualification of examiners, they were recruited as following: two of the examiners were orthopaedic surgeons, two radiologists and two morphologists.

We did, however, not find any personal or profession related bias.

The remarkable part of variations arose from the observations of the same x-ray by the same examiner in different sessions (estimation=6432; 61%; $p<0.0001$) and this is corresponding to the intra-observer variation. To some extent lesser but also statistically significant was the variation between the different examiners (estimation=2693; 26%; $p=0.0015$).

The less is this kind of variation the better is examiners' ability to recognize the object (inter-observer reliability) and to do it repeatedly (intra-observer reliability).

There were no systematic deviations detected amongst the examiners. This means that the variation detected was statistically insignificant ($p=0.2457$). Systematic deviation was not detected if examiner-pairs with different background (orthopaedic surgeon, radiologist, morphologist) were compared.

Variation arising in differentiation and selection of borders of ossificates by computer program Adobe Photoshop was remarkably less than intra- or inter-observer variation. However, the technical error had a statistically significant influence to the sum of errors (estimation=846; $p<0.0001$), but its magnitude was 10 times less than dispersion of diagnosing process.

The option of HO estimation on the plain radiographs can be discussed. In the literature we found two articles demonstrating different situations, where HO is estimated at least by two different x-rays (Moed & Smith 1996, Schmidt & Hackenbroch 1996). These systems demonstrated appearance of HO in regions, which are not detectable on the frontal plane x-rays, but they did not influence proportions of different stages of HO. The important reason of the current study was to improve the reliability of the classification based on the frontal plane x-rays. There were some practical reasons as well – 400 patients,

who underwent total hip replacement during 2002–2003 in the Department of orthopaedic surgery of Tartu University Hospital and who are members of larger prospective study had adequate series of plain x-rays. Into this study were included patients who were operated during the first year (2002) and who passed correctly follow-up.

Similar to our results were achieved in a study where visual scoring method and digital planimetry were applied to measure left ventricular infracted mass in contrast enhanced magnetic resonance imaging. They found the two methods gave in average to the same size of infracted area (19.94%±11.10% with digital planimetry versus 18.92±10.41% with scoring method). However, in this study the technical error dispersion was not calculated exactly (Azevedo Filho et al 2004). In concordance with our results their data are indicating minor significance of the computer aiding in the reducing the total error.

As demonstrated by the analysis of digitalized planimetry, the main source of errors in assessment of severity of HO was the error of recognizing the HO. Similar works have been done also with other x-ray assessment systems. It has been shown, that there was no significant influence of the assessment method, if digital or conventional x-ray techniques were used to diagnose either configuration of hip joint (Essmann *et al* 2006), severity of aseptic loosening after THA (Eklund *et al* 2004) or for applicaton of Larsen classification of rheumatoid arthritis (Young-Min *et al* 2003).

We agree completely with Eklund and co-authors (2004) who pointed out also the need for higher qualificaton of examiners, but it should be emphasized that reliability in practical clinical use would be higher if the assessment system consists of clearly understandable criteria, which need fewer efforts to learn and apply them properly.

Computer aided methods can improve the result, but their usage is still limited (Eklund et al 2004, Essmann *et al* 2006, Young-Min *et al* 2003). Generally physicians need classification systems that exert higher reproducibility, are easier to learn and introduce and have a higher accuracy.

The superiority of Cavalieri principle (Gundersen et al 1988) or digital planimetry for assessment the HO in plain x-rays can be discussed. It has been shown that with complicated objects like bony trabecula the Cavalieri principle has high preciseness and is easy to use (McMillan et al 1999). With much simple objects like wound areas (Langemo et al 1998), cataract dimensions (Datiles et al 1989) or vascular stenosis (Salu et al 2002) differences between the methods to prefer one to another are rather disputable. In the current study those two methods were not compared leaving this question to be answered by the future investigations.

Summarizing these results we may conclude that the diagnostic criteria of estimation system and skillfulness of its use but not the professional background has the main role in arising a diagnostic error.

CONCLUSIONS

In the digital planimetry estimation on the x-rays the main errors appear during diagnostics of HO developing after total hip arthroplasty. Technical error, appearing during image processing by computer program influenced the total error less and subspecialization of doctors did not cause systematic bias.
Thus, the use of digitized planimetry can add only minor additional value in diminishing the estimation error and is rather intended for scientific use than for everyday practice.

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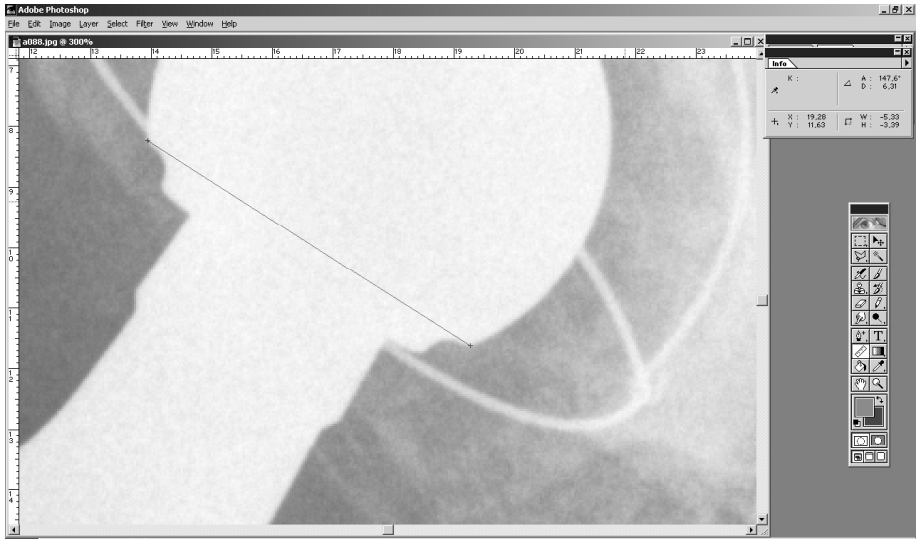
Table 1. Estimation of dispersion components ant their significance.

Covariance Parameter	Standard estimate	Error	p-value	Percent of standard estimate in error component
X-ray **(a_i)**	31416	8859	0.0004	
Technical variation **(b_j)**	598	515	0.2457	5,7%
Inter-observer variation **(c_{ij})**	2693	848	0.0015	25,5%
Intra-observer variation **(d_{ijk})**	6432	769	<.0001	60,9%
Residual **(d_{ijk})**	846	65	<.0001	8,0%

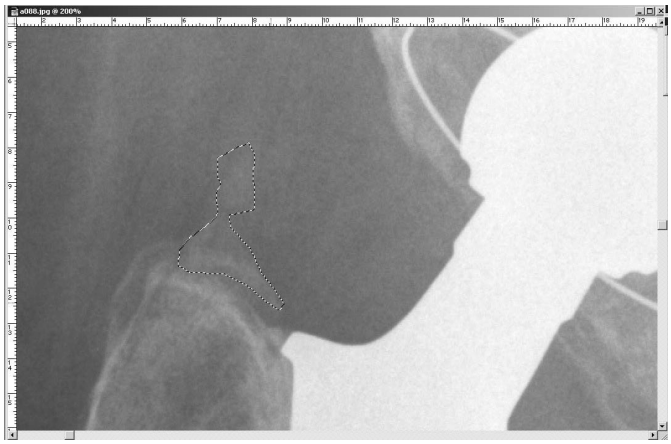
A



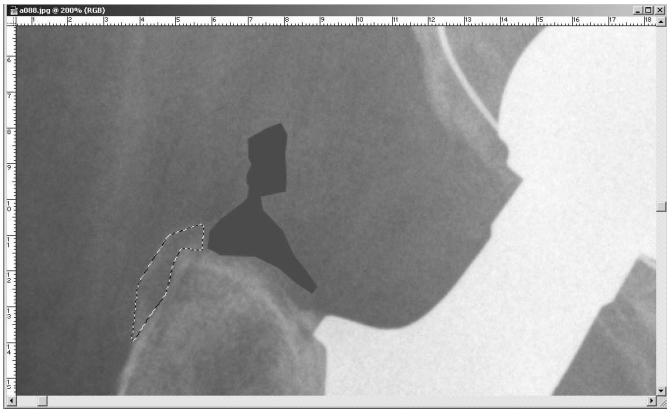
B

Figure 1. Calibration of x-rays using isotropically projected (bilaterally symmetric) measures with total hip prosthesis with known value (distal neck diameter of femoral component and measure of femoral head edge). (A) Calculating pixels' area using the rectangle area that corresponds to the known measures of hip prosthesis. (B)

A



B



C

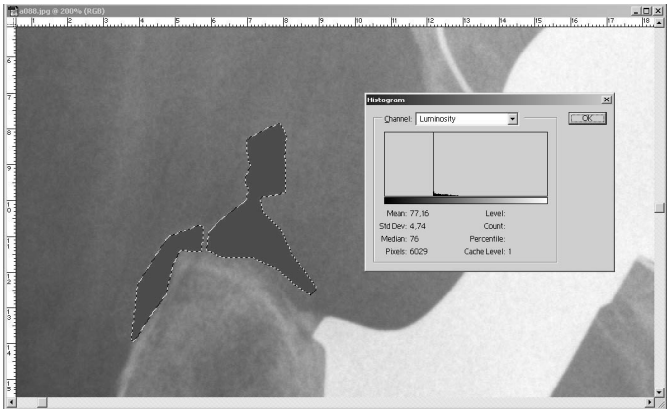


Figure 2. Selection of projection of HO (A), differentiation (B) and digital planimetry measuring a field under the projection (C). All estimators/observers used postoperative x-ray as control.

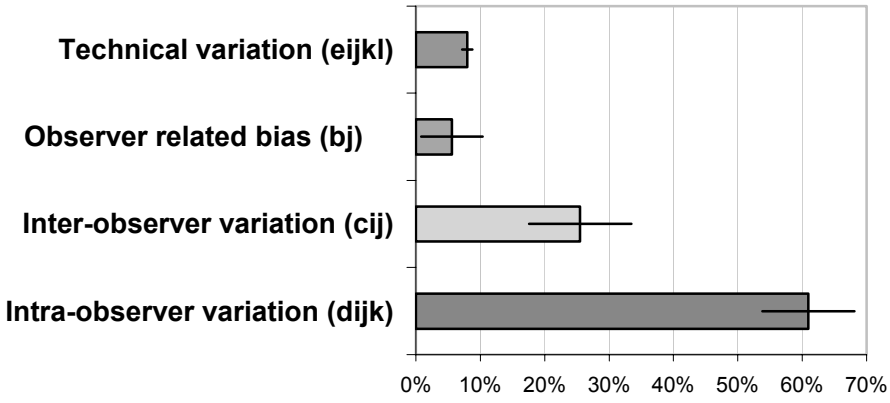


Figure 3. Graphical presentation of error dispersion (statistical significancies are present in the Table 1).

V

Toom A, Arend A, Gunnarsson D, Ulfsparre R, Suutre S, Haviko T, Selstam G.
Bone formation zones in heterotopic ossifications: histologic findings and increased expression of BMP-2, TGF-β_2 and TGF- β_3. *Calcif Tissue Int,* 2007; 80(4):259–67

VI

Toom A, Suutre S, Märtson A, Haviko T, Selstam G, Arend A.
Osteoprogenitor cells for the heterotopic ossification in the experimental rat model
(Submitted to *Acta Orthopaedica*)

OSTEOPROGENITOR CELLS FOR THE HETEROTOPIC OSSIFICATION IN THE EXPERIMENTAL RAT MODEL

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ABSTRACT

Introduction.
It is widely discussed, whether the origin of heterotopic bone after total hip arthroplasty is either bone marrow stromal cells from drilled femoral canal or damage to the surrounding soft tissues causing osteochondral devlopment of multipotent cells.
Methods: An animal model was designed to clarify this problem using standardized muscular damage together with standardized implantation of beta-tricalcium phosphate with or without induction with rhBMP-2. Standardized muscle pinching and capsulotomy were accompanied by drilling of femoral canal in one side. Removed implants were dissected following principles of stereology and were investigated using histological, histomorphometrical and immunohistochemical methods.
Results: Only minimal formation of osteochondral tissue was seen in the saline-immersed implants without significant differences whether femoral canals were opened or not. Bone formation was found in all implants immersed in rhBMP-2 with similar volume on both sides. Bone mineralization was even faster if femoral canal cells were not present – mineralized volume to total volume 18.2% *vs* 12.7% ($p<0.019$), respectively.
Interpretation: Our data suggests that the main cause of ectopic bone formation is the damage of the surrounding tissues. For clinical practice these results indicate priority of the careful tissue handling for avoiding heterotopic ossifications.

INTRODUCTION

Heterotopic ossification (HO) is a common phenomenon after total hip arthroplasty (THA). The incidence has been reported to vary significantly, being as high as 90% (Søballe 1988, Puzas et al. 1989, Ahrengart 1991, Sawyer et al. 1991, Toom et al. 2001). There are probably different reasons for occurrence of HO, but trauma combined with some predisposing factors seems to be a crucial eliciting factor after THA (Stover et al. 1991, Nilsson and Persson 1999).

Different mechanisms have been proposed for the pathogenesis of heterotopic ossification. The idea of required involvement of multipotent bone marrow mesenchymal stem cells in the process of the pathogenesis of HO (Puzas et al. 1987) is apparently as old as the Friedenstein's observation on the ability of bone marrow cells to direct connective tissues to osteogenic development (Friedenstein et al. 1966). Another important factor for HO induction may be traumatic injury of muscles causing haematoma, which was thought to lead to proliferation of the perivascular connective tissue and thereafter deviation to osteochondral development, as postulated in 1958 by Ackermann. Finally, a cellular source as a crucial factor for heterotopic ossification might also be activation of periostal cells with osteogenic capacity as emphasized in 1963 by Urist and McLean.

Chalmers et al (1975) proposed that three conditions are required to occur simultaneously for formation of heterotopic ossification: an inducing agent, a suitable osteoconductive environment, and availability of osteoprogenitor cells. Cohly and the co-workers have from rat experiments concluded that cellular response to tissue damage as well as migration of osteoprogenitor cells from bone marrow may be involved (Cohly et al. 2003). The origin of the osteoprogenitor cells in heterotopic ossification has been extensively discussed. It has been proposed that bone marrow stromal cells together with spread microscopic bone fragments may play a crucial role in heterotopic ossification after total hip arthroplasty (Rumi et al. 2005, Puzas et al. 1987).

Wissler and Vesselinovitch (1968) pointed out that osteogenic potentiality of mesenchymal multipotent cells in the artery walls is similar to mesenchymal multipotent cells of the bone marrow. Later it has been demonstrated that perivascular multipotent cells, i.e pericytes, under controlled conditions can easily be converted into osteochondral development (Brighton et al. 1992, Diaz-Flores et al. 1992, Canfield et al. 1996, Doherty et al. 1998). Hence, presence of a considerable source of multipotent cells with inducible osteogenic potential can be expected everywhere where vasculature is found.

Considering that formation of new bone requires precursor cell proliferation and differentiation into osteogenic lineage, ionizing radiation has been used to prevent HO formation after THA. Based on this knowledge, also an animal model was developed to study preventive measures for HO (Schneider et al. 1998). Using the same model, it was shown in rabbits that irradiation of the

femoral canal resulted in a lower degree of ossification compared to irradiation of the abductor musculature (Rumi et al. 2005).

The aim of the present study was to investigate how the cells originating from the femoral canal influence formation of heterotopic bone.

In order to verify the hypothesis of the basic role of the stem cells and the factors originating from the femoral canal, we designed a model of heterotopic ossification, which allows or restricts access of femoral canal cells to the site of ectopic bone formation, which was induced by implanting osteoconductive matrix with or without an osteoinductive regulatory protein.

MATERIALS AND METHODS

Experimental animals

Twenty 9 months old adult male outbread Bkl: Wistar line rats (purchased from the Scanbur BK AB, Sweden) with body weights 500–600 g were used in this study. The rats were housed in standard cages with 12 hours light/dark cycle at a constant temperature of 21°C and with access to water and standard dry food pellets R70 (Lactamin AB, Sweden) *ad libitum*. Animal care and management, surgical protocol, and procedures were performed in accordance with the routine stated by the Federation of European Laboratory Animal Science Associations and supervised by a licensed veterinarian. This study was approved by the Animal Ethics Committee at the University of Tartu.

Operative procedure and implantation technique

The animals were anesthetized with isoflurane (Isoflurane Baxter®, Baxter Medical AB, Sweden) by inhalation. Antibacterial prophylaxis was performed using a single intramuscular dose of ampicillin prior to the operation. Analgesia was provided before the operation and for 72 hours postoperatively using morphine sulphate. No anti-inflammatory drugs were applied. The method of operation has been described earlier (Toom et al. 2006). In detail, 12–14 mm incision was made over the greater trochanter. The transgluteal approach was used to reach the posterior part of the hip joint capsule. Gluteus maximus was retracted; gluteus medius was pinched for 2 minutes with a standard vascular clamp with a width of 3 mm to produce muscular damage. Bilateral femoral capsulotomy was performed. On the right side the femoral canal was opened just slightly medial from the tip of the greater trochanter. A conic reamer with a maximum diameter of 1.8 mm and an electric drill were used to make the aperture. The opening of the femoral canal was completed manually with a troacar of 1.6 mm in diameter. The tissue remnants were not removed after the opening of the femoral canal. A cube-shaped implant of beta-tricalcium phosphate (ChronOS™ Block, Mathys Medical Ltd, Bettlach, Switzerland) with the size 3.3 x 3.3 x 3.3 mm and with the volume of 36 mm^3 and interconnected

porosity of 70% (being theoretically able to contain approx. 25 mm^3 free liquid) was used. In half of the animals, the implant was immersed in a solution of rhBMP-2 (supplied by prof. Walter Sebald, Biozentrum der Universität Würzburg, Am Hubland, Germany). The estimated amount was 12.5µg/20 µl per implant. Control implants were immersed in vehicle (sterile phosphate-buffered isotonic saline). Implants were placed into the capsulotomy wounds. On the left side the femur was left intact, and care was taken to avoid any periostal injury during the capsulotomy and implantation procedures.

Animals were subdivided into two groups, ten rats in each group, as depicted in Figure 1. In the group 1, where saline-immersed osteoconductive matrix was implanted two subgroups were formed: group 1A – samples taken from right side that had opened femoral canals and group 1B – samples taken from the left side where femur was intact. Similarly in the group 2 animals with osteoconductive matrix immersed in the osteoinductive rhBMP-2 were divided into group 2A – samples taken from right side that had opened femoral canals and group 2B – samples taken from the left side where femur was intact.

X-ray assessment

Correct implant situation was assessed and its *in vivo* dimensions were estimated immediately before the euthanasia using x-ray apparatus (Arman, Russia) and phosphor-plates (Figure 2).

Histologic and morphometric analyses

Euthanasia was performed by decapitation under sedation with isoflurane 3 or 21 days after operation. The implants and samples from the surrounding soft tissues were fixed in neutral buffered formalin. After decalcification with EDTA, histological sections were made according to the principle of systematic uniform random selection (Gundersen 2002). Sections with a thickness of 5 µm for surface analysis according to Cavalieri's principle (Gundersen et al. 1988b) and sections with a thickness of 40 µm for surface/cell counting were collected systematically after every 200 µm. These sections were subjected to systematic uniform random selection (Gundersen 2002) resulting in 7–8 sections per sample for final analysis. The sections were stained with haematoxylin-eosin, azan and toluidine.

Cell counting was performed according to the optical dissector principle (Gundersen et al. 1988a) with the light microscope "Olympus BX51" (Olympus, Olympus Company Ltd., Japan) and analysis software "Cast 2" (Olympus, Olympus Company Ltd., Japan). Applying Cavalieri's principle relative and absolute volumes of different types of tissues in the implants were calculated using a correction factor obtained by in vivo measurements on x-rays to eliminate the tissue shrinkage effect. Also, the surface density and proportions of eroded and osteoid surface were measured in the region of formed bone. All

the morphometry abbreviations used in this paper were based on the standardized bone histomorphometry nomenclature (Parfitt et al. 1987).

Immunohistochemical analysis
The deparafinized sections were treated with 0.6% H_2O_2 to inactivate endogenous peroxidase and then with 1% BSA to block nonspecific binding. After blocking, sections were incubated with primary antibodies either with mouse monoclonal antibody to osteonectin (Acris Antibodies GmbH, Germany), mouse monoclonal antibody to osteocalcin (Abcam Ltd., United Kingdom), or rabbit polyclonal anti-Collagen type I antibody (Research Diagnostics Inc., NJ, USA) for 2 hours at 4ºC. Visualization of the primary antibodies was performed using commercial kit "Strept ABComplex/HRP Duet Mouse/Rabbit system" (Dako Cytomation Denmark A/S, Denmark) and DAB+ Chromogen (Dako Cytomation,USA) for substrate.

Statistical calculations
Sample size was calculated for a power of 0.80 on the alpha level of 0.05.
Calculations of difference were performed using the t-test for paired and unpaired data, and z-test, considering the p-level less than 0.05 significant.

RESULTS

There was no mortality, no postoperative infections or metabolic complications among the experimental animals.

After 3 days in all implants the pores were mostly filled with few inflammatory cells. There was no significant difference between the implants in the degree of cellular penetration. Also, some colonies of connective tissue cells with the low differentiation were presented, mostly in outer pores of implants. Their number was statistically different between the rhBMP-2 (group 2) and saline treated (group 1) implants: 5.22±1.67% and 1.31±0.35%, respectively, ($p=0.004$). There was no obvious difference between the implants where the femoral canal cells were present or absent in respect of their later cellular consistence. Histological investigation demonstrated in the group 2 numerous low differentiated connective tissue cells localized close to vessels in connective tissues surrounding the implants, whereas in the group 1 such cellular activation was not evident (Figure 3). There was no difference between the groups 1A/2A and between 1B/2B.

After 21 days no bone tissue was formed in groups 1A and 1B, except for one implant from group 1B where an osteoid region with a volume of 0.075 mm^3 was detected (Figure 4). In the other samples, osteoblast-like cells were only occasionally found on the surface of the implant, but without any recognizable osteoid or bone formation. Negligible fibrous cartilage formation

was detected with metachromatic staining in the outer pores of all samples of groups 1A and 1B, but there was no statistical difference between the groups. There was also no difference between group 1A and 1B in the number of fibrous tissue cells, capillary sprouts and inflammatory cells. The implants were mostly surrounded by cell-rich fibrous connective tissue with fibers in arbitrary directions, which were observed similarly in both groups. There was no qualitatively distinguishable difference between the osteocalcin and osteonectin expression patterns as revealed by immunohistochemical staining, either.

Heterotopic bone was induced in all implants in the groups 2A and 2B. Ossification occurred around the implants, in the pores of implantsand also the implant itself was partly replaced by newly formed bone (Figure 5). Complete replacement of the implant occurred in one case of five in the group 2A and in two cases of five in the group 2B. A rigid bridging between the implant and the greater trochanter was seen in three cases of five in the group 2A and in four cases of five in the group 2B. There was no case of completely ossified bridging, but dominant consistence was calcified fibrocartilage or hyaline cartilage.

The mean ratio of bone volume to total sample volume (BV/TV) was similar for the groups 2A and 2B, 33.1% (SD 7.4%) vs 30.0% (SD 7.6%), respectively ($p=0.234$). However, comparison of groups' 2A and 2B revealed that more bone had been mineralized in the group 2B, where the mineralized volume (Md.V/TV) was higher: 18.2% (SD 4.5%) vs 12.7% (SD 2.9%) ($p=0.019$). Osteoid volume to bone volume (OV/BV) was higher in the group 2A, 57.3% (SD 5.0%) vs 45.3% (SD 3.0%), than in the group 2B ($p=0.010$). The ratio of osteoid surface to total bone surface (OS/BS), being 61.3% (SD 5.8%) for group 2A and 56.8% (SD 6.8%) for group 2B, did not differ significantly between the groups ($p=0.184$). The ratios of osteoblast surface to bone surface (Ob.S/BS) or the ratio of eroded surface to bone surface (ES/BS) did not differ significantly between the groups 2A and 2B.

Distribution of different tissue types is summarized in the Fig 6.

Immunohistochemical study revealed no difference between the localization of the osteocalcin and the localization of osteonectin that could be distinguished by qualitative analysis. Staining for collagen I was used to increase the preciseness of the osteoid differentiation. Taken together, there was no significant bone formation either in the presence (group 1A) or in the absence (group 1B) of femoral canal cells during a relatively short time period (21 days) unless an additional osteoinductive factor was applied.

DISCUSSION

Many ectopic bone formation models have been developed in animals in order to investigate the mechanisms of HO formation, but also for investigation of different tissue engineering products. Mostly, subcutaneous (Kantorowitz et al. 1990, Jiang et al. 2005, Kim et al. 2005, Kroese-Deutman et al. 2005, Liu et al. 2005) or intramuscular pouches (Wang et al. 1999, Vogelin et al. 2000, Nakagawa et al. 2003, Liang et al. 2005) have been used to achieve ectopic bone formation.

Our method was developed to study heterotopic bone formation under standardized conditions regarding bone induction and osteoconduction using homogenous materials with an exact chemical composition (beta-tricalcium phosphate, rhBMP-2). Our previous experience with establishment of HO in rats, using perforation of the femoral canal and leaving the debris created by drilling *in situ* in the capsulotomy wound (unpublished data), resulted in a very low rate of HO formation, even less than 50%. Therefore, we introduced a model of heterotopic ossification model using exogenous implants where bone induction was controlled by exogenous osteoinductive substance, which allowed to elucidate the influence of bone marrow stem cells on heterotopic ossification processes more precisely.

Another reason was better to mimic the situation after total hip replacement surgery. There are many reports on occurrence of HO in the region of the abductor musculature, especially in the gluteus medius and gluteus minimus muscles (Kjaersgaard-Andersen et al. 1990, Ahrengart 1991, Bisla et al. 1976, Søballe et al. 1988, Nollen and Slooff 1973, Puzas et al. 1989), which corresponds to the structures often subjected to tensile forces and damaged during the total hip replacement surgery. The same localization of HO was used also in our model. We used localization close to the greater trochanter, immediately under the abductor musculature, and in connection with the capsulotomy wound.

The rat heterotopic bone formation model by Schneider and co-workers (1998), which mimics the real situation after hip replacement surgery, has a disadvantage in the variable degree of bone induction. The method of Kantorowitz and co-authors employs a non-physiological situation where HO is created in a subcutaneous localization (Kantorowitz et al. 1990), which can be avoided with our method. By determining the exact degree of the osteoconductive and/or osteoinductive factors, our method allows to diminish the number of subjects included in assessment of different local factors.

We used exogenous rhBMP-2 to standaridze the osteoinductive signal. Osteoinductive properties of exogenous BMP-2 have been well evidenced (Wozney et al. 1988, Urist 1965).

Reason for choosing beta-tricalcium phosphate as the carrier for rhBMP-2 was its property to form a depot of recombinant BMP-2, as was shown by

Uludag and co-authors (1999) as well as by other authors (Seeherman and Wozney 2005). This avoids strong dilution and uncontrolled concentrations of rhBMP-2 in the implant region as well as its systemic effects. Moreover, it is well known that beta-tricalcium phosphate possesses excellent osteoconductive properties (Seeherman and Wozney 2005).

In several studies the purpose has been assessment of the risk factors for HO. It has been found in association with hypertrophic osteoarthritis, contralateral total hip replacement, previous hip surgery, and subtrochanteric femoral osteotomy, as well as lateral or anterolateral approach, very high body mass index, repeated surgery and male gender (Vastel et al. 1998, Nilsson and Persson 1999, Toom et al. 2001, Eggli and Woo 2001). Furthermore, also direct damage of surrounding muscles (Puzas et al. 1989, Nilsson and Persson 1999), as well as extended exposure to mechanical factors in the conditions of long operative times have been shown to be a risk factor for HO (Toom et al. 2001). The above data support the idea that local damage induce HO.

Heterotopic bone formation without exogenous bone induction
Minimal osteoid formation was recorded in a limited localization in one subject from group 1B (Figure 4). Considering that in this case the femoral canal cells were absent, this kind of osteoinduction has to be applied on local factors and osteoprogenitor cells activated by local trauma.

Heterotopic bone formation with exogenous bone induction with rhBMP-2
After induction larger areas of implants were penetrated by the low differentiated connective tissue cells at 3 days. Later, bone formation occurred first on the outer surface of the implant. No polarity of ossification was detected in the implants, which is indicative of cell migration from all directions into the implant. Evidently the cells originated equally from the surrounding muscles, vessels and from the joint capsule as well as from the femoral canal in the group 2A. This is highly suggestive of the hypothesis that osteoprogenitor cells responsible for heterotopic bone formation around the proximal femur originate from the surrounding soft tissues like the joint capsule, the blood vessels, the tendon- and the muscle sheets, and probably also from the periostal pool. Also, the histologic finding of “wandering” perivascular cells in surrounding tissues of implants 3 days after induction support this hypothesis. Our results may explain a prospective randomized study in arthroplasty patients where pulsative irrigation was used to remove bone dust (containing osteogenic cells) from the femoral canal did not find any difference between the treated and control groups (Sneath et al. 2001). Moreover, a clinical study showed that the gluteus minimus debridment had a positive effect in decreasing the incidence of HO after acetabular fractures (Rath et al. 2002). However, this study involved no adequate control group.

In our study osteoid volume (OV/BV) was very high in both group, 2A and 2B, which indicates a high rate of formation of new bone. Thus, the HO formation was evident on both hind limbs in all animals. It is difficult to explain the significantly lower mineralized volume of bone (Md.V/TV) on the right side, while the volume of formed bone (BV/TV) itself was similar on both sides. This may be due to an increased washout of rhBMP-2 caused by bleeding from the femoral canal. Yet some inhibitory factors originating from the femoral canal cannot be ruled out.

CONCLUSIONS

When the surrounding muscular tissues and the joint capsule were injured in similar way, there were no differences in the intensity of bone formation between the groups in which femoral canal cells were either present or absent. Moreover, faster calcification of formed bone was detected in the group with the absence of the femoral canal cells. This indicates, that the main cause of heterotopic ossification can be damaged surrounding tissues.

Presence of femoral canal cells does not seem to exert an additional osteoinductive or osteogenic effect as revealed by the absence of differences between groups 1A and 1B. Moreover, in the presence of strong osteoinductive signal (local rhBMP2 treatment), cells or substances originating form femoral canal may be even inhibitory to mineralization processes in newly formed bone.

For clinical practice, we recommend to handle the surrounding highly vascularized tissues with care. Bone debris and bone marrow cells eliberated by the reaming of acetabulum and the femoral canal seem to be of minor importance.

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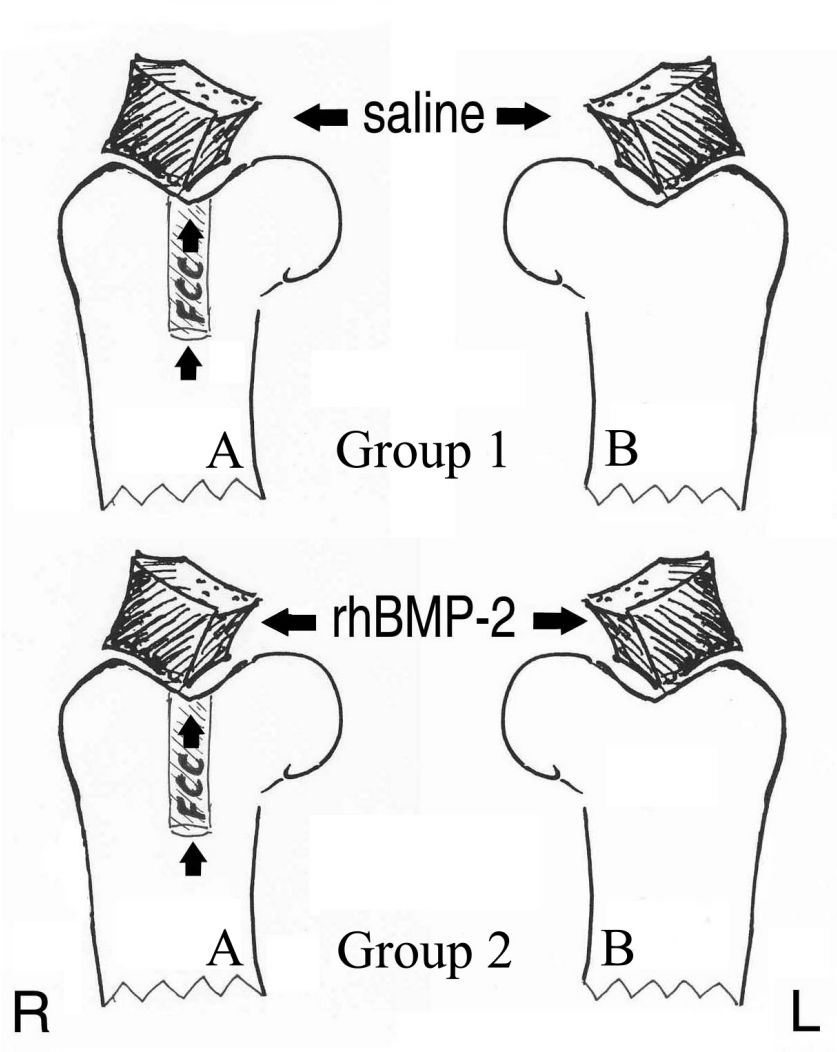


Figure 1. Experimental design – peculiarities of different sites and groups.

R – right side; L – left side; FCC – femoral canal cells; rhBMP-2 – recombinant human bone morphogenetic protein-2.

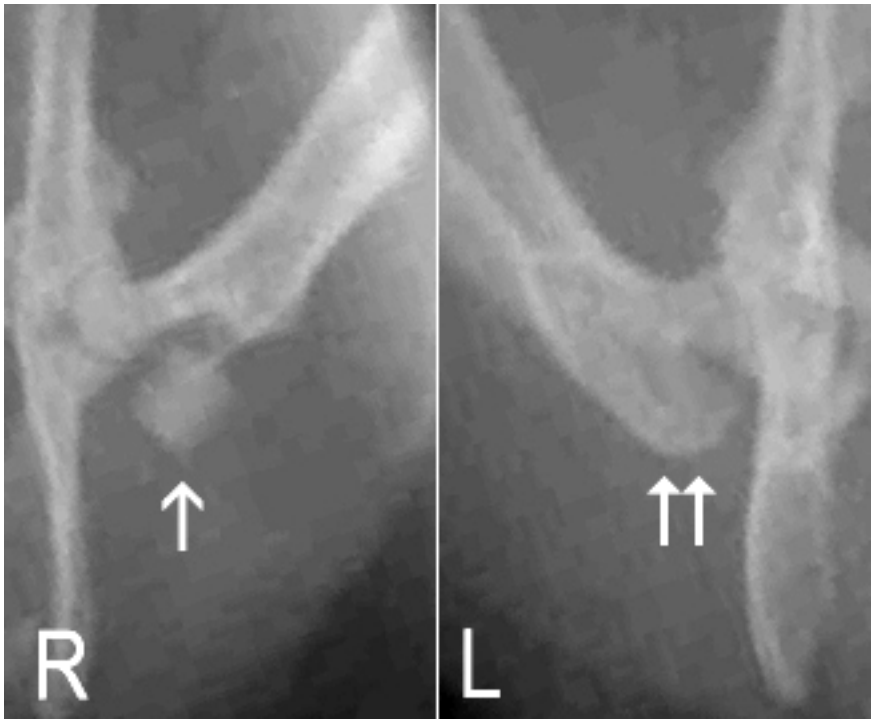


Figure 2. Pelvic x-ray of an animal receiving rhBMP-2 treated implants on both sides. Note the marked change in the quadrilateral shape of the implant on the left side (double arrows) where the femur was left intact, as well as the consolidation between the implant and the greater trochanter. On the right side (single arrow), where the femoral canal was opened, the implant is much less surrounded by newly formed bone and consolidation is less prominent.

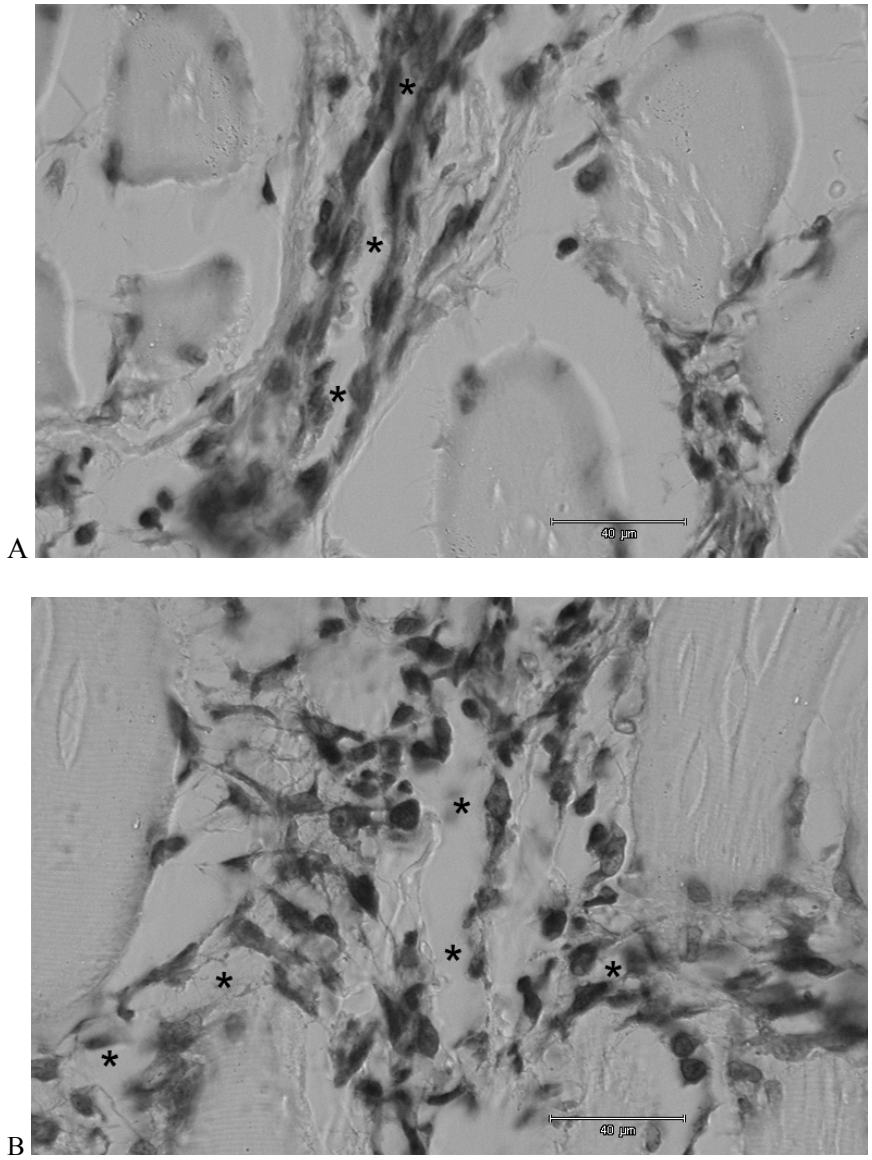


Figure 3.
A. Perivascular cells are well oriented and close to the vascular walls in surrounding tissues of the implants without induction in the groups without osteoinduction. Vascular lumen (*). Staining with toluidine.
B. Perivascular cells are enlargened, orientation is diverse respect the direction of vascular lumen and there is evidence of cellular translocation apart from the blood vessels. Representative finding for the groups with osteoinduction. Vascular lumen (*). Staining with toluidine.

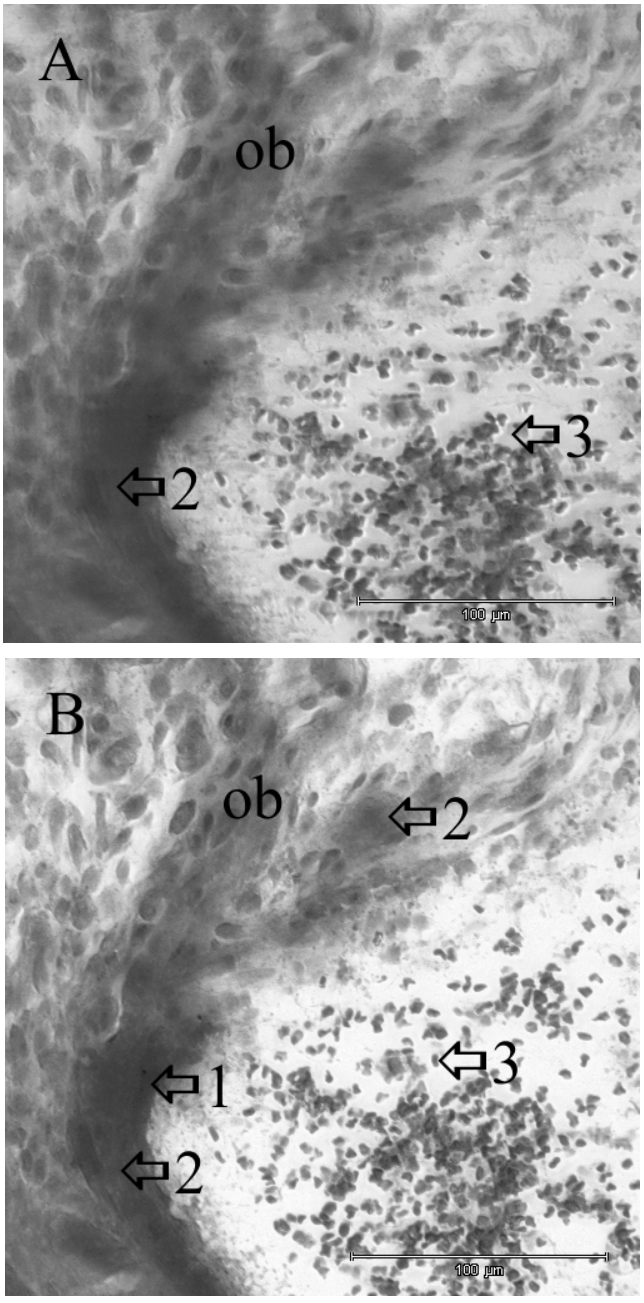
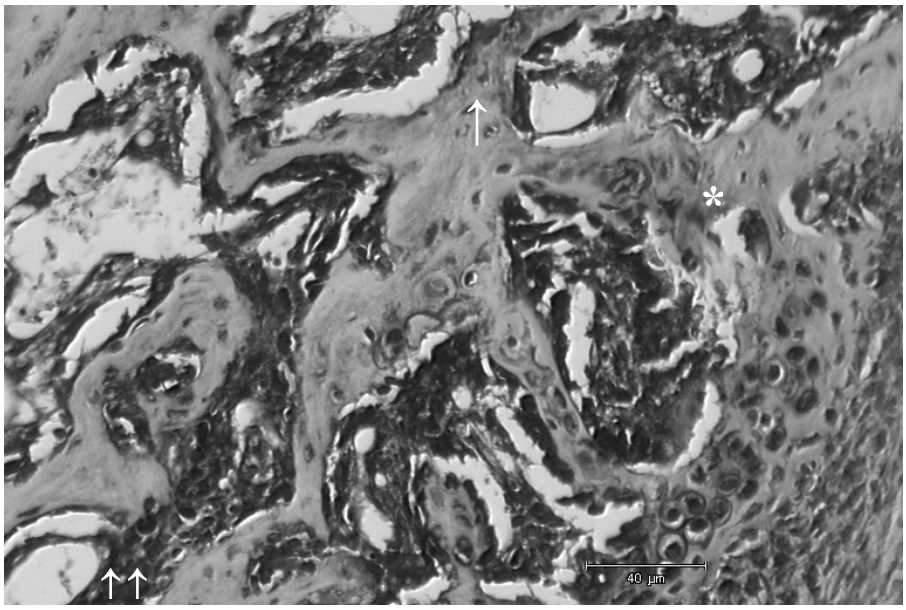
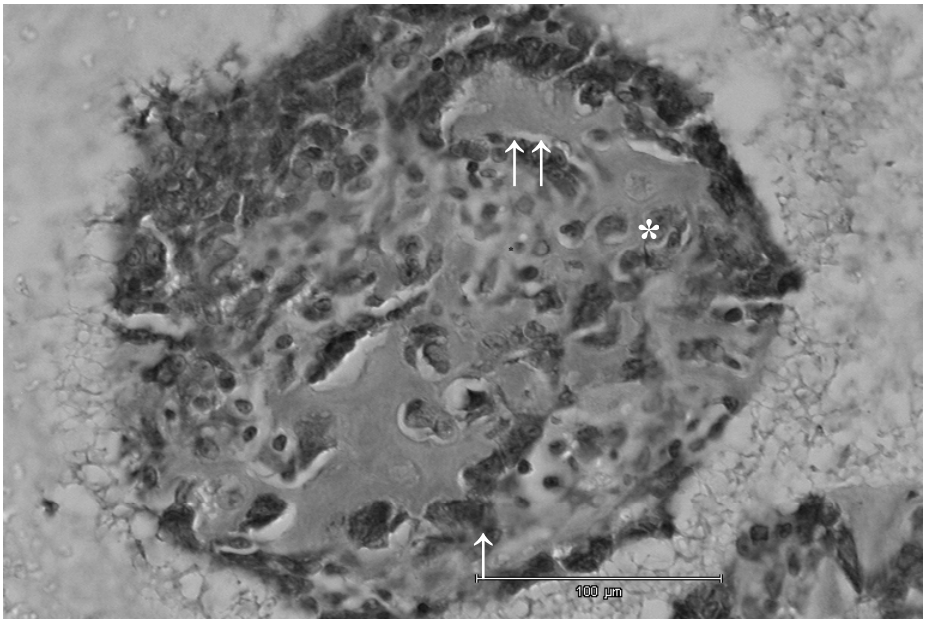


Figure 4. There was only one limited area in one sample from group 1A where osteoid formation was recognizable.
A (3D picture in red-green print-code, red-green glasses should be used to capture the 3D effect), and
B (summary of 3D-picture, AZAN stain): 1 – Osteoid mineralization, 2 – osteoid seal, 3 – implant, note some inflammatory cells inside the implant material, ob – osteoblastic cells.

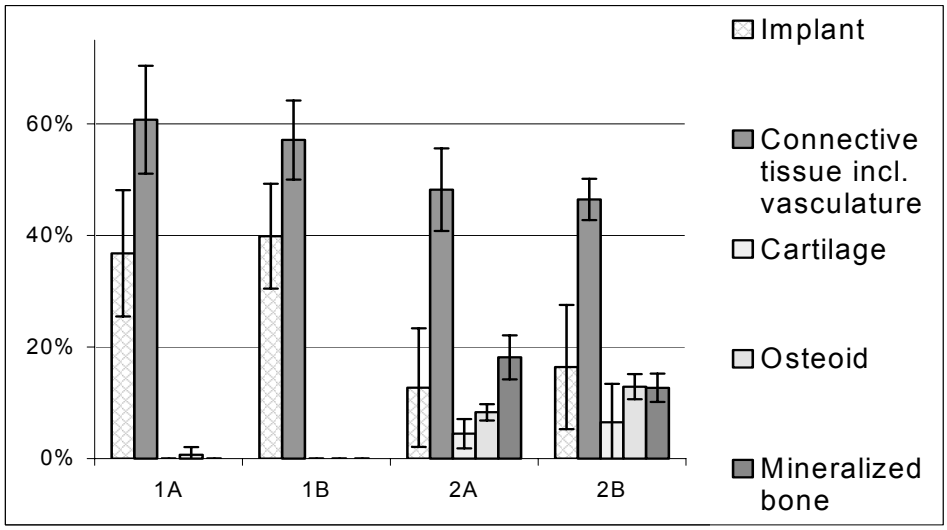


5A. Immunohistochemical staining for osteocalcin. Note very fast bone formation expressed as intense resorption of the implant material by osteoclasts (↑), newly formed bone trabeculae (↑↑) and marked osteocalcin expression (brown stain) in actively synthesizing osteoblasts but also in osteocytes and in some cartilage cells present in cartilage remnants (*) (Osteocalcin, counterstain with toluidin).



5B. Note the remodelling of implant structure expressed as osteoclastic resorptive activity (↑), as well as the formation of cartilagineous (*) and bony structures inside the pores (↑↑); (toluidin stain).

Table 1. Volumes of different tissues and the implant after 21 days accordingly to histomorphometric analysis following the Cavalieri principle.



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Scientific work

Main field of research:
- heterotopic ossification after total hip arthroplasty;
- use of bioimplants to enhance the bone formation;
- posttraumatic bone repair.

26 publications, including 15 in last five years and 7 publications in international peer-reviewed journals.

Voluntary activities:

2006–... International Cartilage Repair Society (ICRS), member
2005–... European Orthopaedic Research Society (EORS), member
2001–... Estonian Orthopaedic Society, member
2000–… Estonian Junior Doctors Society, member
2004–2006 University of Tartu, member of council of the medical faculty

Advanced training

1. AO symposium on Basic Fracture Care, Laulasmaa 2007
2. EORS Annual Meeting, Bologna 2006
3. EAMST symposium and round-table on bone grafting, Lisbon 2005
4. Laboratory animal course, FELASA, Tartu 2005
5. Stereology course, University of Tartu, Centre of Molecular and Clinical Medicine, Tartu 2004
6. From cellular structure to function. Course at advanced light microscopy, Tartu-Turku 2003
7. Seminar-Workshop: Mobile Bearing Knee Artroplasty, Tallinn 2002
8. AO Seminar on Shaft fractures. Intramedullary nailing, Tallinn, 2001
9. C.F.P.-Hip & S.T.A.R. Ankle Joint Symposium of the Tartu University Clinics, Clinic of Traumatology and Orthopedics, Estonia, 2001

Awards

DePuy Award for young orthopaedic surgeon 2006
Scientific competition for Estonian students 2000, diploma.
Scientific conference for students of Tartu University Medical Faculty in 1999, I award.
Scientific conference for students of Tartu University Medical Faculty in 1998, I award.

CURRICULUM VITAE

Alar Toom

Sündinud: 1. detsember 1974 Tartus
Kodakondsus: Eesti
Aadress: L.Puusepa 8, Tartu 51014, Eesti;
Telefon: +372 7 318 282; +372 53401143
e-mail: alar.toom@ut.ee

Haridus

2005–...	SA Tartu Ülikooli Kliinikum, arst-resident ortopeedia erialal
2001–2007	Tartu Ülikool, arstiteaduskond, arstiteaduse doktorantuur
2006–2006	Tartu Ülikool, arstiteaduskond, biomeditsiini magistrantuur, eksternina, kaitsmine 27.09.2006
2000–2001	Tartu Ülikool, arstiteaduskond, internatuur
1993–2000	Tartu Ülikool, arstiteaduskond, arstiteaduse eriala
1982–1993	Tartu 8. Keskkool

Teenistuskäik

2007–...	Tartu Ülikool, Traumatoloogia ja ortopeedia kliinik, ortopeedia teadur
2005–...	SA Tartu Ülikooli Kliinikum, arst-resident ortopeedia erialal
2004–2007	Tartu Ülikool, Traumatoloogia ja ortopeedia kliinik, spetsialist ja erakorraline teadur
2001–2005	Tartu Ülikool, arstiteaduskond, arstiteaduse doktorant
2000–2001	Tartu Ülikool, arstiteaduskond, arst-intern.

Teadustöö

Peamised uurimisvaldkonnad.

- Heterotoopne ossifikatsioon puusaliigese endoproteesimise järgselt
- Bioimplantaatide kasutamine luutekke soodustamiseks
- Luumurru reparatsioon

Kokku 26 publikatsiooni, viimasel viiel aastal 15, sh 7 artiklit rahvusvahelistes eelretsenseeritavates ajakirjades.

Muu teaduslik organisatsiooniline ja erialane tegevus:

2006–...	International Cartilage Repair Society (ICRS), liige
2005–...	European Orthopaedic Research Society (EORS), liige
2001–...	Eesti Traumatoloogide-Ortopeedide Selts, liige
2000–…	Eesti Nooremarstide Ühendus, liige
2004–2006	Tartu Ülikooli arstiteaduskonna nõukogu, doktorantide esindaja

Erialane enesetäiendus

1. AO sümpoosium luumurdude ravist, Laulasmaa 2007
2. EORS-i aastakoosolek, Bologna 2006
3. EAMST sümpoosium ja ümarlaud luuplastikast, Lissabon 2005
4. Katseloomateaduse kursus, FELASA, Tartu 2005
5. Stereoloogiakursus, Tartu Ülikool, Kliinilise ja molekulaarse meditsiini keskus, Tartu 2004
6. Raku struktuurist funktsioonini. Edasijõudnute kursus valgusmikroskoopiast, Tartu-Turu 2003
7. Seminar-töötuba: liikuva platooga põlveliigese artroplastika, Tallinn 2002
8. AO seminar luumurdude ravist. Intramedullaarne naelastamine, Tallinn 2001
9. C.F.P. puusa- & S.T.A.R hüppeliigese artroplastika kursus, Tartu Ülikooli Kliinikumi rahvusvaheline kursus, Tartu 2001

Tunnustused

DePuy noore ortopeedi preemia – 2006
Haridusministeeriumi teadustööde konkurss üliõpilastele, diplom – 2000
Arstiteaduskonna üliõpilaste teadusliku konverentsi I koha preemia – 1999
Arstiteaduskonna üliõpilaste teadusliku konverentsi I koha preemia – 1998

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

1. **Heidi-Ingrid Maaroos.** The natural course of gastric ulcer in connection with chronic gastritis and *Helicobacter pylori*. Tartu, 1991.
2. **Mihkel Zilmer.** Na-pump in normal and tumorous brain tissues: Structural, functional and tumorigenesis aspects. Tartu, 1991.
3. **Eero Vasar.** Role of cholecystokinin receptors in the regulation of behaviour and in the action of haloperidol and diazepam. Tartu, 1992.
4. **Tiina Talvik.** Hypoxic-ischaemic brain damage in neonates (clinical, biochemical and brain computed tomographical investigation). Tartu, 1992.
5. **Ants Peetsalu.** Vagotomy in duodenal ulcer disease: A study of gastric acidity, serum pepsinogen I, gastric mucosal histology and *Helicobacter pylori*. Tartu, 1992.
6. **Marika Mikelsaar.** Evaluation of the gastrointestinal microbial ecosystem in health and disease. Tartu, 1992.
7. **Hele Everaus.** Immuno-hormonal interactions in chronic lymphocytic leukaemia and multiple myeloma. Tartu, 1993.
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114. **Triine Annus**. Allergy in Estonian schoolchildren: time trends and characteristics. Tartu, 2005.
115. **Tiia Voor.** Microorganisms in infancy and development of allergy: comparison of Estonian and Swedish children. Tartu, 2005.

116. **Priit Kasenõmm.** Indicators for tonsillectomy in adults with recurrent tonsillitis — clinical, microbiological and pathomorphological investigations. Tartu, 2005.
117. **Eva Zusinaite.** Hepatitis C virus: genotype identification and interactions between viral proteases. Tartu, 2005.
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119. **Tiina Stelmach.** Epidemiology of cerebral palsy and unfavourable neurodevelopmental outcome in child population of Tartu city and county, Estonia Prevalence, clinical features and risk factors. Tartu, 2006.
120. **Katrin Pudersell.** Tropane alkaloid production and riboflavine excretion in the field and tissue cultures of henbane (*Hyoscyamus niger* L.). Tartu, 2006.
121. **Külli Jaako.** Studies on the role of neurogenesis in brain plasticity. Tartu, 2006.
122. **Aare Märtson.** Lower limb lengthening: experimental studies of bone regeneration and long-term clinical results. Tartu, 2006.
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128. **Priit Põder.** Clinical and experimental investigation: relationship of ischaemia/reperfusion injury with oxidative stress in abdominal aortic aneurysm repair and in extracranial brain artery endarterectomy and possibilities of protection against ischaemia using a glutathione analogue in a rat model of global brain ischaemia. Tartu, 2006.
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