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BONE QUALITY AND MESENCHYMAL STROMAL CELL CAPACITY IN TOTAL HIP REPLACEMENT

Significance for stem osseointegration measured by radiostereometric analysis

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Jessica J. Alm

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University of Turku
Faculty of Medicine
Department of Orthopaedics and Traumatology
Turku Doctoral Program for Molecular Medicine (TuDMM)
Turku University Hospital , Orthopaedic Research Unit, Turku, Finland

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Supervised by

Professor Hannu T. Aro, MD, PhD
Department of Orthopaedics and Traumatology
University of Turku and Turku University Hospital
Turku, Finland

Docent Teuvo A. Hentunen, PhD
Department of Cell Biology and Anatomy
Institute of Biomedicine
University of Turku
Turku, Finland

Reviewed by

Professor Mikko Lammi, PhD
Department of Integrative Medical Biology
Umeå University
Umeå, Sweden

Associate professor Charles R. Bragdon, PhD
Department of Orthopaedic Surgery
Massachusetts General Hospital
Harvard Medical School
Boston, Massachusetts, USA

Opponent

Professor Julie Glowacki, PhD
Department of Orthopedic Surgery
Brigham and Women's Hospital,
Harvard Medical School
Boston, Massachusetts, USA

Cover: Background photograph of human mesenchymal stromal cells in culture by Jessica J Alm. Schematic drawing of human proximal femur with a total hip prosthesis with RSA tantalum markers on the implant and in the surrounding bone by Niko Moritz

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“I am among those who think that science has great beauty. A scientist in the laboratory is not a mere technician: he is also a child placed before natural phenomena which impress him like a fairy tale.”

Marie Curie

ABSTRACT

Jessica J Alm

Bone quality and mesenchymal stromal cell capacity in total hip replacement: Significance for stem osseointegration measured by radiostereometric analysis

University of Turku, Faculty of Medicine, Department of Orthopaedics and Traumatology, Turku Doctoral Programme of Molecular Medicine (TuDMM), Turku University Hospital, Orthopaedic Research Unit, Turku, Finland. Annales Universitatis Turkuensis, Medica-Odontologica, 2016, Turku, Finland

Immediate implant stability is a key factor for success in cementless total hip arthroplasty (THA). Cementless techniques were originally designed for middle-aged patients with normal bone structure and healing capacity, but indications have expanded to also include elderly patients. Poor local bone quality, as a result of osteoporosis (OP), and age-related geometric changes of the proximal femur, may jeopardize initial implant stability and lead to increased migration of the implant components thereby compromising biological fixation and osseointegration. Mesenchymal stromal cells (MSCs) are essential in the process of osseointegration. Age-related dysfunction of MSCs is suggested to be a main contributory factor in altered bone repair with aging and therefore may influence osseointegration. The hypothesis of this prospective clinical study was that preoperative bone quality and MSC capacity dictate stability and osseointegration of femoral stems in cementless THA, especially in women after menopause.

A total of 61 consecutive women (age <80 yrs) scheduled for cementless THA for primary hip osteoarthritis (OA) were screened for undiagnosed primary or secondary OP, vitamin D insufficiency and other metabolic bone diseases. Prior to THA, patients underwent aspiration of iliac crest bone marrow for analysis of MSC capacity using optimized isolation and culturing protocols. All patients received a cementless total hip implant with an anatomically designed hydroxyapatite (HA) coated femoral stem and ceramic-ceramic bearings. Per-operative biopsy of the intertrochanteric bone was taken for *ex vivo* analysis of the local cancellous bone quality using micro-CT imaging and biomechanical testing. After surgery, stem migration and osseointegration was monitored for two years using radiostereometric analysis.

The majority of women with hip OA was osteopenic or osteoporotic. These conditions were associated with increased periprosthetic bone loss in the proximal femur and impaired initial stability and delayed osseointegration of the femoral stem. Altered intraosseous dimensions of the proximal femur, as well as aging, also had adverse effects on initial stem stability and were associated with delayed osseointegration. Local bone mineral density of the operated hip and the quality of intertrochanteric cancellous bone had less influence than expected on implant migration. The THA females showed differences in the osteogenic properties of their MSCs. Patients with MSCs of low *in vitro* osteogenic capacity displayed increased stem subsidence after the initial 3 months settling period and thereby delayed osseointegration.

The results suggest that decreased skeletal health, such as low systemic BMD and decreased osteogenic properties of bone marrow MSCs, has major influence on early stability and osseointegration of cementless hip prostheses in female patients.

Keywords: Bone quality; Mesenchymal stromal cells; Cementless total hip arthroplasty; Radiostereometric analysis; Osseointegration; Bone mineral density; DXA; Osteoporosis

TIIVISTELMÄ

Jessica J Alm

Luun laadun ja mesenkymaalisten kantasolujen toiminnan vaikutus lonkan tekonivelen paranemiseen

Turun yliopisto, Lääketieteen tiedekunta, Ortopedia ja traumatologia, Molekyylilääketieteen tohtoriohjelma, Turun Yliopistollinen keskussairaala, Ortopedian tutkimusyksikkö
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Tekonivelleikkaus on erinomainen toimenpide lonkan nivelrikon hoidossa. Jos leikkausmenetelmäksi valitaan biologisesti kiinnittyvä tekonivel, olennaisinta on saavuttaa komponenttien välitön stabiliteetti. Se mahdollistaa uudisluun kasvun implantin karhennetulle pinnalle. Ikääntymiseen liittyvä luuston haurastuminen ja reisiluun yläosan ydinontelon laajentuminen voivat heikentää tekonivelen komponenttien tukevuutta ja näin hidastaa niiden kiinnittymistä luuhun. Tällainen on mahdollista erityisesti naisilla vaihdevuosien jälkeen. Näiden potilaiden yksilölliset erot luun parantavien solujen (mesenkymaalisten kantasolujen) määrässä ja toiminnassa voivat osaltaan vaikuttaa heidän tekoniveltensä kiinnittymisnopeuteen.

Tähän prospektiiviseen kliiniseen tutkimukseen osallistui 61 naispotilasta, joille tehtiin sementöimätön lonkan tekonivelleikkaus nivelrikon takia. Ennen leikkausta potilaille tehtiin seulontatutkimukset osteoporoosin ja muiden luuston aineenvaihduntasairauksien tunnistamiseksi. Leikkauksen yhteydessä potilailta otettiin luuydinnäyte suoliluusta, josta analysoitiin mesenkymaalisten kantasolujen jakautumis- ja erilaistumiskyky luunsoluiksi. Leikkauksen aikana otettiin näyte reisiluun yläosan hohkaluun hienorakenteen ja mekaanisten ominaisuuksien arvioimiseksi. Leikkauksen jälkeen tekonivelen reisikomponentin kolmiulotteista migraatiota ja kiinnittymistä seurattiin radiostereometrisellä analyysillä (RSA) 2 vuoden ajan.

Valtaosalla potilaista oli alentunut luuntiheys (osteopenia tai osteoporoosi). Osteopeenisillä ja osteoporoottisilla potilailla todettiin kiihtynyttä luukatoa tekonivelen reisikomponentin ympärillä sekä komponentin lisääntynyttä migraatiota ja hidastunutta kiinnittymistä. Reisiluun yläosan ydinontelon laajentuminen ja potilaan korkea ikä lisäsivät reisikomponentin migraatiota, mutta reisiluun hohkaluun laatu ei vaikuttanut migraation määrään. Potilailla, joilla todettiin mesenkymaalisten kantasolujen alentunut kyky erilaistua luusoluiksi *in vitro*, todettiin reisikomponentin lisääntynyttä migraatiota ja hidastunutta kiinnittymistä.

Tulokset osoittavat, että ikääntymiseen liittyvät luustomuutokset ja yksilölliset erot mesenkymaalisten kantasolujen määrässä ja toiminnassa voivat osaltaan vaikuttaa lonkan tekonivelten paranemiseen naisilla vaihdevuosien jälkeen.

Avainsanat: Mesenkymaaliset kantasolut, lonkan tekonivelleikkaus, radiostereometrinen analyysi (RSA), osseointegraatio, DXA-luuntiheysmittaus, osteoporoosi

SAMMANFATTNING

Jessica J Alm

Inverkan av benkvalitet och mesenkymala stamcellskapaciteten på inläkningen av cementfria höftledsproteser

Åbo Universitet, Medicinska fakulteten, Enheten för ortopedi och traumatologi, Molekylärmedicinska doktorandprogrammet (TuDMM), Åbo Universitetscentralsjukhus, Ortopediska forskningsenheten

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Total höftledsplastik är en framgångsrik behandling för återskapande av förlorad funktion och lindring av smärta vid höftledsartros. Vid användning av cementfria lårbenskomponenter, som är konstruerade för biologisk fixering, är det av yttersta vikt att uppnå omedelbar mekanisk stabilitet för att möjliggöra benvävnadens inväxt i implantatets yta och därmed en långvarig fixering. Med stigande ålder ökar skelettets sköret och lårbenskanalen vidgas, vilket kan försvaga stabiliteten av lårbenskomponenter och därmed fördröja fixeringen till benvävnaden. Detta är framförallt troligt hos kvinnor efter övergångsåldern, vilka utgör majoriteten av höftledsplastikpantienterna idag. Hos denna patientgrupp kan ålders- och menopaus-relaterade förändringar i antal och kvalitet på benvävnadens stamceller (mesenkymala stam celler) ytterligare fördröja inläkningen av höftledsproteser.

Den här prospektiva kliniska studien inkluderade 61 kvinnliga patienter som genomgick cementrfri höftledsplastik för höftledsartros. Före opertionen genomgick patienterna omfattande screening för osteoporos samt andra benmetaboliska sjukdomar. I samband med operationen togs ett benmärgsprov från höftbenskammen för analys av den mesenkymala cellpopulationens förmåga att proliferera och differentiera till benceller. Under operationen togs även en benbiopsi från lårbenets övre del för analys av den trabekulära benvävnadens mikrostruktur och mekaniska egenskaper. Efter höftledsplastiken gjordes uppföljande mätningar av lårbenskomponenternas tredimensionella mikromigration med hjälp av radiostereometrisk analys (RSA). Uppföljningstiden var 2 år.

Majoriteten av patienterna diagnostiserades med låg bentäthet (osteopeni eller osteoporos). Hos dessa patienter konstaterades en ökad förlust av benvävnaden runt lårbenskomponenten samt en ökad migration och fördröjd fixering av komponenten. Vidgad lårbenskanal och högre ålder var förknippade med ökad komponentmigration, medan kvaliteten på lårbenetes trabekulära benvävnad inte påverkade migrationen. Hos de patienter vars mesenkymala celler uppvisade en försämrad bendifferentieringsförmåga i cellodling konstaterades en ökad migration och fördröjd fixering av lårbenskomponenten.

Resultaten påvisar att åldersrelaterade förändringar i den skeltala hälsan, så som ökad benskörhet och försämrad bendifferentieringsförmåga hos benvävnadens stamceller, inverkar på den tidiga läkningsprosessen av höftledsproteser hos kvinnor efter övergångsåldern.

Nyckelord: Benkvalitet, mesenkymala (stam)celler, höftledsplastik, osteoporos, radiostereometrisk analys (RSA), DXA bentäthetsmätning, osseointegrering

CONTENTS

ABBREVIATIONS

AA	Ascorbic acid-2-phosphate
ABG-II	Anatomic Benoist Girard II
ALP	Alkaline phosphatase
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenetic protein
BMU	Basic multicellular unit
BTMs	Bone turnover markers
βGP	β-glycerophosphate
CFI	Canal flare index
CFU	Colony formation unit
CI	Confidence interval
CN	Condition number
COL1	Type 1 collagen
COX-2	Cyclooxygenase-2 / Prostaglandin-endoperoxide synthase 2
CTX	C-terminal cross-linked telopeptide of collagen type I
CV	Coefficient of variation
DA	Degree of anisotropy
Dex	Dexamethasone
DXA	Dual-energy absorptiometry
EBRA	Ein Bild Röntgen Analyse
FHL2	Transcriptional modulator four and a half LIM-only protein
GCs	Glucocorticoids
HA	Hydroxyapatite
HHS	Harris hip score
HSC	Haematopoietic stem cell
IGF	Insulin-like growth factor
IL	Interleukin
µCT	Micro computed tomography
ME	Mean error of rigid body fitting
MSCs/hMSCs	Mesenchymal stem/stromal cells/human MSCs
MTPM	Maximum total point motion
NPCs	Non-collagenous proteins
NTX	N-terminal cross-linked telopeptide of collagen type I
OA	Osteoarthritis
OB	Osteoblast
OC	Osteoclast
OCN	Osteocalcin
OP	Osteoporosis
OPG	Osteoprotegrin
OSX	Osterix
PD	Population doubling
PINP	N-terminal propeptide of collagen type I
Pi	Inorganic phosphate
PPi	Pyrophosphate
PTH	Parathyroid hormone
RANKL	Receptor activator of nuclear factor-κβ ligand
RIA	Radioimmunoassay
RSA	Radiostereometric analyses
RUNX2	Runt family transcription factor 2
S-AFOS	Serum levels of bone specific alkaline phosphatase
SMI	Structure model index
TFs	Transcription factors
TGF-β	Transforming growth factor β
THA	Total hip arthroplasty
TRACP-5b	Tartrate resistant acid phosphatase, isoform 5b
UI	Uncoupling index
WOMAC	Western Ontario and McMaster Universities Osteoarthritis Index

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-VI).

I The incidence of osteopenia and osteoporosis in women with hip osteoarthritis scheduled for cementless total joint replacement.
Mäkinen TJ, **Alm JJ**, Laine H, Svedström E, Aro HT
Bone 2007; 40: 1041-1047

II Transient 100 nM dexamethasone treatment reduces inter- and intraindividual variations in osteoblastic differentiation of bone-marrow derived human mesenchymal stem cells.
Alm JJ, Heino TJ, Hentunen TA, Väänänen HK, Aro HT
Tissue Eng C 2012; 18: 658-666

III Female patients with low systemic BMD are prone to bone loss in Gruen zone 7 after cementless total hip arthroplasty. A 2-years DXA follow-up of 39 patients.
Alm JJ, Mäkinen TJ, Lankinen P, Moritz N, Vahlberg T, Aro HT
Acta Orthop. 2009; 80: 531-537

IV Quality of intertrochanteric cancellous bone as predictor of femoral stem RSA migration in cementless total hip arthroplasty.
Moritz N, **Alm JJ**, Lankinen P, Mäkinen TJ, Mattila K, Aro HT
J Biomech. 2011; 44: 221-227

V Low BMD affects initial stability and delays stem osseointegration in cementless total hip arthroplasty in women. A 2-years RSA study of 39 patients.
Aro HT, **Alm JJ**, Moritz N, Mäkinen TJ, Lankinen P
Acta Orthop. 2012; 83: 107-114.

VI *In vitro* osteogenic capacity of bone marrow MSCs from postmenopausal women reflect the osseointegration of their cementless hip stems
Alm JJ, Moritz N, Aro HT
Bone Reports 2016; 5: 124-135

1 INTRODUCTION

Total hip arthroplasty (THA) is one of the most successful medical interventions, recognized as an effective and reliable treatment for degenerative joint disease. Millions of patients are being operated annually worldwide, with a global trend over the last decade towards increased use of cementless fixation, representing the majority of THAs in the Western world (Sadoghi et al., 2012).

Cementless THA relies on biological fixation of the implant to the surrounding bone, a process considered to take 3-6 months. Osseointegration is defined as ingrowth and/or ongrowth of new bone to the implant surface (Branemark et al., 1977, Albrektsson et al., 1981). Major part of the biological process depends on availability and function of mesenchymal stromal cells (MSCs) (Deschaseaux et al., 2009, Davies, 2003). Upon implantation, the prosthesis gets in contact with resident and invading MSCs, and under proper mechanical conditions the outcome depends on the ability of MSCs to proliferate and differentiate. If appropriate stem cells are not present, osteogenesis is inhibited (Kienapfel et al., 1999, Goodman et al., 2009). Age-related dysfunction of MSCs is emerging as the main cause of bone loss and altered repair with aging (Marie and Kassem, 2011, Baker et al., 2015). Decreased MSC capacity with age is well documented *in vitro*, but it is unclear how this reflects *in vivo* bone healing of the donors.

Initial stability achieved at time of surgery is a prerequisite for successful biological fixation. This is affected by implant related factors (design, chemical composition, surface topography, coatings), surgical technique, and the quality of the surrounding bone bed (Khanuja et al., 2011, Engh et al., 1987). Excessive micromotion and gaps at the interface restrain cellular processes and inhibit osteogenesis, which may delay or inhibit osseointegration (Pilliar et al., 1986, Daugaard et al., 2011, Vandamme et al., 2011). Radiostereometric analysis (RSA) is a unique method allowing monitoring progression of implant osseointegration in arthroplasty patients. The method provides accurate and precise measurement of 3-dimensional implant micromotion relative to bone (Kärrholm et al., 1997, Valstar et al., 2005). Osseointegration is characterized by cessation of micromotion (Mann et al., 2012) and by applying RSA the time point for osseointegration can be identified.

Although cementless THA was originally developed for middle-aged patients with normal or close to normal bone quality, current indications include also aged patients (Dutton and Rubash, 2008, Troelsen et al., 2013). Today, postmenopausal women constitute a majority of cementless THA patients (Sadoghi et al., 2012, Mäkelä et al., 2010). Based on the traditional view of an inverse relationship between osteoarthritis (OA) and osteoporosis (OP) where OA was considered to protect against OP, there have been no indications to screen for decreased BMD in postmenopausal females with hip OA. During THA however, it is not uncommon to encounter large differences in cancellous bone of these patients. It appears that the prevalence of OP in these patients has been clearly underestimated (Glowacki et al., 2003), and they may exhibit qualitative and quantitative variations in their MSCs.

Despite the increasing number of cementless THAs performed, very little is known about biological factors actually affecting osseointegration of long-bone prostheses. The impact of osteoporosis on osseointegration of cementless long-bone implants has not been systematically studied in patients. Although MSCs are considered essential in the process, it is unclear how intrinsic properties such as osteogenic differentiation and ability to lay down mineralized matrix correlate with the outcome of an arthroplasty. RSA technology has revolutionized how osseointegration can be monitored. Still, RSA has not been applied to prospectively monitor early implant migration and osseointegration of cementless femoral stems in postmenopausal women at risk of decreased systemic bone quality.

This doctoral thesis was initiated to investigate the impact of bone quality on the healing of cementless THA in postmenopausal women with primary hip osteoarthritis. The first objective was to investigate the skeletal health, with emphasis on systemic BMD, in the female patients scheduled for cementless THA. Using RSA, osseointegration was monitored for 2 years after surgery and the impact of preoperative systemic and local bone quality parameters, including bone marrow MSCs, were investigated. Although the study population is relatively small, it still represents the largest female population of cementless THA that have been studied with RSA. The impact of MSC capacity on osseointegration of cementless THA has never been investigated before.

2 REVIEW OF THE LITERATURE

2.1 CEMENTLESS TOTAL HIP ARTHROPLASTY (THA)

2.1.1 Background and incidence of THA

Total hip replacement is considered one of the most successful surgical interventions, providing rapid and substantial pain relief and enhanced quality of life for patients suffering from diseased hip joints (Bozic et al., 2011, Lavernia and Alcerro, 2011). Also in terms of cost-effectiveness, THA is regarded as one of the greatest medical successes (Pulikottil-Jacob et al., 2015). Both cemented and cementless fixations of THA provide good outcomes today. The global trends show increasing annual numbers of THAs, expanded age-range of the target group and increasing popularity of cementless fixation solutions.

The original concept of THA was to replace diseased hip joints, mainly due to degenerative osteoarthritis (OA), in patients aged between 60 and 75. The THAs were estimated to last for approximately 10 years and was not believed to meet the more demanding physical activity of younger patients. Primary hip OA is the main reason for THA, accounting for 80-90% of all primary THAs (Sadoghi et al., 2012). Although age indication has expanded as the THA procedure has evolved, patients between 65 and 80 years is the largest age group globally (Troelsen et al., 2013, Sadoghi et al., 2012, Wyatt et al., 2014). Women represent over 55% of THA patients (Sadoghi et al., 2012, Kurtz et al., 2014), reflecting the higher incidence of hip OA in women after menopause (Dagenais et al., 2009).

Primary candidates for THA are patients with degenerative OA, which causes unacceptable pain and limitations to everyday life that is not relieved by non-surgical treatments. THA effectively provides pain relief and improved function, improved walking ability, sleep, and social function, usually within three months after surgery (Bozic et al., 2011, Lavernia and Alcerro, 2011). Favorable results from patients over 80 years (Pieringer et al., 2003, Jämsen et al., 2014) and 90 years (Pagnano et al., 2003) demonstrate that age should not be a limiting factor for THA.

The annual number of THAs performed worldwide has constantly increased during the last 20 years (Pivec et al., 2012, Tsertsvadze et al., 2014) and is estimated at 130 procedures per 100 000 inhabitants. In Finland the annual number of THAs has doubled during the last 20 years (Finnish National Institute of Health and Welfare statistical report 2013 www.thl.fi). With increasing number of older individuals and a generation expecting good functional activity and independency, the annual demand for THA is expected to increase exponentially (Pivec et al., 2012, Fehring et al., 2010). In parallel more patients will outlive their THAs due to the increased life expectancy and improved general health of elderly. Therefore, good long-term outcomes have to be improved and secured. This includes identification of all parameters affecting the success of THA, not only traditional implant-related factors. Especially in the elderly patient population, bone quality and turnover are decisive.

BOX 1. Modern history of THA

The modern THA techniques developed from the early 1960s when Sir John Charnley (1911-1982) introduced fixation of THA with polymethyl methacrylate (PMMA) cement on large scale. The hip replacement procedure turned out to be a bigger success than expected and as the first generation of cemented THAs (implanted 1965-1975) showed excellent short-term results, indications were expanded to include also younger patients. But high fracture rates and aseptic loosening resulted in 10-year revision rates of 10-30%. Improvement in design, cementing and surgical techniques, instruments and sterility contributed to improved results of the second generation cemented THAs (implanted late 1970s and 1980s). Long-term follow-up of the Charnley prosthesis have shown impressive 20-25 years survival rates of 80-96% (Caton and Prudhon 2011), and 30-78% survival at 30-35 years (Callaghan et al., 2009).

The high 10-20 years revision rates of the first generations cemented THAs due to loosening was especially common in younger patients (Cornell et al., 1986), and was related to bone resorption and osteolysis due to cement particles (Harris et al., 1976). To avoid this "cement-disease", new fixation solutions where developed aiming at a direct bond between the prosthesis and bone without the use of cementing. The first report on cementless arthroplasty was published in 1979 (Lord et al., 1979).

2.1.2 Cementless THA – Original concept and current indication

The modern THA techniques are based on the pioneering work by Sir John Charnley (1911-1982) (**Box 1**). Cementless fixation of total hip prosthesis was developed to overcome problems associated with cement as fixation. Successful long-term fixation of cementless THAs rely on the surrounding bone creating a biological anchoring of the implant. Deposition of bone tissue onto the implant surface without interfering fibrous tissue results in a firm osseous fixation, a phenomenon known as osseointegration.

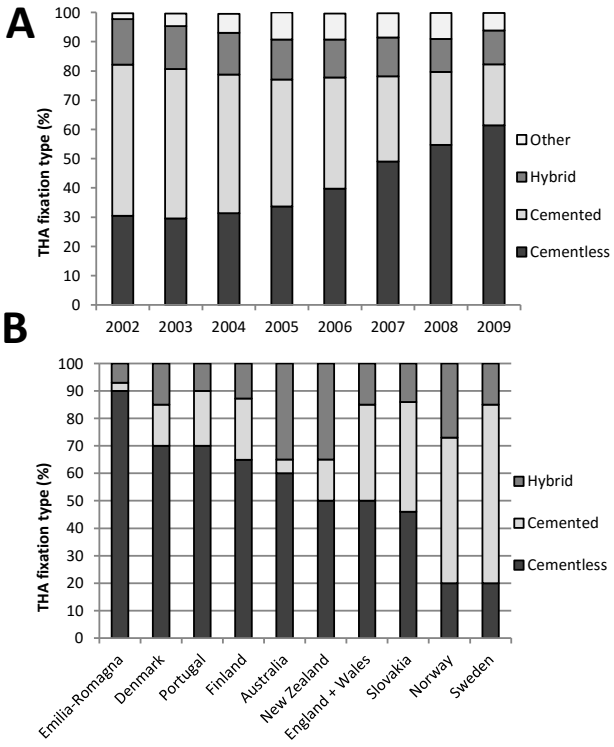

FIGURE 2.1 *Fixation type distribution of primary total hip arthroplasty (A) in Finland over time (2002-2009), and (B) comparison of fixation type distribution of primary total hip arthroplasty in different countries in the year 2009. Finnish data from the Finnish National Institute of Health and Welfare (www.thl.fi), international data adopted from Sadoghi et al (2012).*

The cementless technique was developed for younger patients with good bone quality. Against the initial indications to choose cementless implants for younger and cemented implants for older patients, the original target group has been extended to include also elderly patients with good bone quality (Dutton and Rubash, 2008). Most stem designs today show excellent long-term results and cementless fixation is growing as the leading technique for THA worldwide (Khanuja et al., 2011, Mellon et al., 2013), in all age groups due to the growing population of physically active elderly and extended life time expectancy (Dutton and Rubash, 2008, Mäkelä et al., 2010). In Finland cementless THA has increased from 30% of all THAs in 2002 to 61% in 2009 (www.thl.fi)(**Figure 2.1A**). The trend is similar in other European countries, even if the trend has been slow in Sweden and Norway (Sadoghi et al., 2012, Troelsen et al., 2013, Wyatt et al., 2014) (**Figure 2.1B**). In US, 60-90% of THAs are estimated to be cementless (Khanuja et al., 2014).

CEMENTLESS FEMORAL COMPINENTS IN ELDERLY

Concerns regarding cementless femoral stems in elderly patients relates to the risk of decreased initial fixation due to unfavorable geometric changes of the proximal femur, low bone quality (Dorr et al 1997) and an increased risk of failed bone ingrowth (Dorr et al., 1997) due to decreased osteogenic potential (Bellantuono et al., 2009).

Many studies have demonstrated excellent long-term results of cementless femoral stems in younger patients but cementless fixation in elderly is still a topic of debate (Dutton and Rubash, 2008). Register and retrospective studies have shown comforting 8-15 years results of cementless THAs of different designs in patients over 55 years (Oosterbos et al., 2004, Mäkelä et al., 2010), patients over 65 years (Butler et al., 2005, Kirsh et al., 2001), over 70 years (Rhyu et al., 2012, Sporer et al., 2004, Berend et al., 2004), and even in patients over 80 years (Pieringer et al., 2003, Keisu et al., 2001, Jämsen et al., 2014) (**Table 2.1**). A study of the Finnish arthroplasty register reported better 10-15 years survival of cementless compared to cemented THA in patient aged 55-74. In patients over 75 years the long-term survival was equal for cemented and cementless THAs (Mäkelä et al., 2008). In contrast, a systematic review of register studies reported a lower general 10-15 years survival rate of cementless THAs regardless of age (Corbett et al., 2010). Notably, this review covered studies published between 2000 and 2010, with probably high representation of early cementless stem designs. More recent mid-term (5 to 10 years) follow-up studies on cementless THA of different designs in patient populations with mean ages of 65 - 75 years (range up to 90) demonstrate excellent clinical and radiological outcomes, with 96-100% 10 year stem survival to aseptic loosening (Omlor et al., 2010,

Callary et al., 2012, Sariali et al., 2012), also in patients with osteoporosis (Rhyu et al., 2012) and altered proximal femur morphology (Dalury et al., 2012, Kim et al., 2013).

Age per se should not be considered a contraindication for cementless THA, since beneficial effects are similar or even better in the age population. However, comorbidities are associated with higher revision incidence (Dorr et al., 1990, Ong et al., 2010). With the extended age indication, candidates for cementless THA include patient groups with potentially higher prevalence of low bone quality, bone disorders and comorbidities affecting bone formation and implant fixation. Long-term studies of cementless stems in over 80 year old patients are still limited in number and the effect of low bone quality and compromised bone forming capacity on osseointegration has not been systematically evaluated.

FAILURE AND REVISION

Despite high survival rates of both cemented and cementless THAs today, revision surgeries represents 11-18% of all THAs (Kurtz et al., 2007, Pivec et al., 2012). The overall 10 year revision risk (cemented and cementless together) based on different national registers is estimated to 5-20% (Corbett et al., 2010). With cementless THAs of modern designs failure rate has decreased significantly but revisions of failing implants are still a major concern. Revisions are surgically demanding and for the individual patient, every revision procedure is a major incident contributing to reduced health state (Bozic et al., 2011).

TABLE 2.1 Cementless THA in aged patients

Authors	Year	Stem design	n (F/M)	Age (range)	Patient profile/ Dorr classification	Follow-up (range)	Stem survival
Kirsh et al.,	2001	Proximally HA-coated	69 (34/38)	74 (65-85)	>65 years of age OP Singh index	7 years (2-10)	100%
Reitman et al.,	2003	Tapered porous	62 (35/27)	70 (>65)	Dorr A-B n=29 Dorr C n=33	13.2 years (>10)	98.4%
Pieringer et al.,	2003	Alloclassic SL	43	83 (80-92)	>80 years of age	5 years (3-8 years)	100%
Sporer et al.,	2004	Modular proximally coated	122 (74/48)	77 (70-90)	>70 years of age	5 years (2-8.5)	100%
Berend et al.,	2004	Double-tapered Proximally coated	47 (34/13)	79 (>75)	Dorr A n=10 Dorr B n=27 Dorr C n=10	5 years	98%
Kelly et al.,	2007	Proximally HA-coated	15 (7/8)	54	Dorr Type C	11.5 years (9-14)	100%
Sanz-Reig et al.,	2011	Proximally coated tapered	149 (76/73)	71 (65-88)	Dorr A n=96 Dorr B n=47 Dorr C n=6	13 years (10-15)	99%
Stroh et al.,	2011	Proximally coated tapered	33 (17/18)	85 (80-90)	>80 years of age	4 years (2-7.5)	97%
Rhyu et al.,	2012	Diverse	40 (27/13)	79 (70-95)	T-score -3.2 (-2.5 to -4.8)	1 year	100%
Dalury et al.,	2012	Proximally coated tapered	53 (18/35)	67 (39-88)	Dorr Type C	6 years (4-9)	100%
Kim et al.,	2013	Short metaphyseal-fitting anatomic	230 (101/129)	65 (31-81)	Dorr A n=72 Dorr B n=73 Dorr C n=85	5.6 years (5-7)	100% 100% 98%
Yuasa et al.,	2016	Diverse	30 (All F)	83 (80-89)	>80 years of age	5.6 years (1-10)	100%

n; number of patients; F female; M male

2.2 BONE BIOLOGY

Understanding bone and its regulators at the tissue, material and cellular levels is crucial for better understanding the process of cementless hip implant fixation and factor affecting osseointegration.

2.2.1 Bone tissue and composition

The bony environment is a biologically active system of cellular functions and molecular regulators contributing to the continuous process of bone turnover. In addition to pivotal mechanical (protection, stabilization and locomotion), biological (site for hematopoiesis) and physiological (calcium and phosphorous homeostasis) functions, bone is a far-reaching metabolic endocrine organ (Chapurlat and Confavreux, 2016). Bone has an impressive ability to repair and it is therefore not surprising that bone tissue contain bone-regenerative stem cells.

Two types of bone tissue are defined based on histological porosity and microstructure. **Cortical** (compact) bone with a well-organized structure and high density constitutes approximately 80% of the total bone mass, while **trabecular** (cancellous or spongy) bone with an irregular structure, lighter weight, and high porosity constitute 20% (**Figure 2.2**). Microscopically cortical and trabecular bone can be woven (immature) or lamellar (mature) based on the arrangement of the collagen network. Macroscopically the basic structure of any bone segment comprise an outer layer of compact bone (cortex), overlaying trabecular bone, and an inner medullary cavity (**Figure 2.2**). The proportion between cortex and medulla varies with skeletal site and segment, and with gender and age (Buckwalter et al., 1996). The outer surface of the cortex is covered by the periosteum, a two layer membrane. The outer layer is fibrous, populated by undifferentiated MSCs and fibroblast-like cells and is connected to the surrounding soft tissues. The inner layer (cambium layer) is populated by committed progenitors, has osteogenic potential and produce new bone through periosteal apposition.

Bone extracellular matrix consists to 60-70% of an inorganic mineral phase mainly (95%) composed of a calcium phosphate mineral analogous to crystalline hydroxyapatite (HA), and an organic phase composed of collagen (18-25%), water (5-10%) and non-collagenous proteins (NCPs) and proteoglycans (2%) (Robey and Boskey, 2008). Cells constitute only 2-5% of the total bone tissue. The mineral phase harbors up to 99% of body calcium, 85% of phosphorus and 40-60% of sodium and magnesium (Glimcher, 1987).

***Collagen type I* (COL1)** accounts for about 90% of the organic matrix, providing the framework of bone matrix essential for maintaining the structure of bone tissue and is required for proper mineralization. NCPs constitute 10% of the organic bone matrix. Most NCPs have multiple functions in organization of the extracellular matrix, mediating cell-matrix and matrix-mineral interactions, and regulating mineralization. NPCs regulate bone cells by binding growth factors to the local environment (Robey and Boskey, 2008, Allori et al., 2008b).

Alkaline phosphatase (ALP) is a glycoprotein enzyme highly abundant in bone and one of the molecular hallmarks of bone formation. This bone isoform of tissue-nonspecific ALP is a membrane-bound enzyme but can be secreted in membrane vesicles during mineralization. ALP promotes mineralization by hydrolyzing the inhibitor pyrophosphate (PPi) into inorganic phosphate (Pi) required for mineralization (Golub, 2011). Its enzymatic activity requires Zn^{2+} and Mg^{2+} as catalysts and high pH (8-10) (Harris, 1990). ALP is mostly expressed by osteoblasts, but also by young osteocytes, progenitors and chondrocytes (Martin et al., 2013). ALP expression is enhanced by 1,25-dihydroxyvitamin D_3 and decreased by supraphysiological levels of glucocorticoids and parathyroid hormone (PTH) (Majeska and Rodan, 1982).

Osteocalcin (OCN) is one of the more abundant NCPs in bone. It is a small protein (5 kDa) produced mainly by mature osteoblasts in the late stage of bone remodeling. OCN binds to HA with high affinity in a vitamin K-dependent manner (Robey and Boskey, 2008). The biological functions of OCN are still unclear, with several regulatory functions suggested. In bone, OCN regulate both mineralization (Desbois and Karsenty, 1995) and osteoblast and osteoclast activity (Glowacki et al., 1991, Neve et al., 2013). Expression of OCN is upregulated by vitamin D and repressed by high levels of glucocorticoids (Beresford et al., 1984). Other NCPs include ***osteonectin*, *osteopontin*, and *bone sialoprotein***.

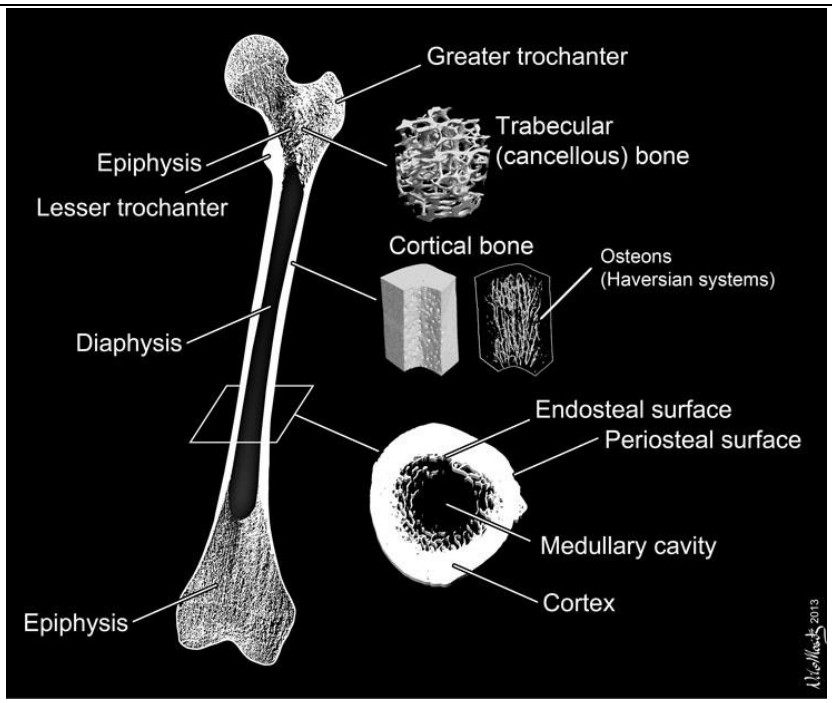

FIGURE 2.2 *Macroscopic and microscopic structure of the human femur. Illustration by Niko Moritz.*

2.2.2 Bone cells

Osteoblasts (OBs) and osteoclasts (OCs) are considered the main bone cell types due to their functions as bone forming and bone resorbing cells, respectively. However, recent findings emphasize major roles for osteocytes and bone lining cells, especially in regulating OBs, OCs, and bone turnover. In addition, the newly identified osteal macrophages (osteomacs) has been added to the family of bone cells.

Osteoblasts (OBs) are the principal bone forming cells, derived from MSCs through a stepwise differentiation cascade. They reside in groups on the bone surface producing osteoid in a coordinated process (**Figure 2.3**). OBs use a range of membrane-bound and released factors for communicating with each other and with other bone cells (Allan et al., 2008). In addition to collagen type I and NCP synthesis, production of ALP is an essential part of OB function. OBs also produce a range of regulatory molecules essential for directing bone turnover, OCs, and their own activity. OBs and other osteoblast lineage cells regulate OC differentiation, activity and survival by producing macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor-κβ ligand (RANKL), and the RANKL decoy receptor osteoprotegrin (OPG) (Kular et al., 2012, Martin et al., 2013). By the end of a remodeling sequence, OBs lose their osteoid forming capacity and become bone lining cells, or become trapped within the bone matrix and differentiate into osteocytes or undergo apoptosis (50-70%)(Lynch et al., 1998).

Bone lining cells are differentiated from OBs and cover the majority of endosteal and trabecular bone surfaces that are not being remodeled, possibly to protect against OCs between remodeling sequences (Kular et al., 2012)(**Figure 2.3**). Bone lining cells express hormone receptors and produce growth factors and cytokines similar to OBs, and are part of the complex signaling network regulating bone turnover (Martin et al., 2013). Osteoclast formation can possibly activate bone lining cells to expose the mineralized bone and prepare the surface for resorption.

Osteocytes are terminally differentiated OBs embedded in bone during bone formation (**Figure 2.3**). Osteocytes are smaller than OBs, have lost many of their organelles and have low bone forming activity. Mature osteocytes are identified by expression of matrix extracellular phosphoglycoprotein (MEPE), dentine matrix protein-1 (DMP-1), fibroblast growth factor 23 (FGF-23) and sclerostin (Bonewald, 2011). Osteocytes are

the most abundant and long lived bone cell, representing over 90% of bone cells with a life span up to 25 years (Atkins and Findlay, 2012). Embedded throughout bone, osteocytes lie within lacunae separated from each other, with long cellular processes extending through the bone matrix forming an extensive network for communication with each other and with bone lining cells and OBs at the bone surface (Dudley and Spiro, 1961, Bonewald, 2011). Through this network osteocytes act as mechano-sensors responding to mechanical stress, microdamage, injury, disuse and unload, estrogen deficiency and therapeutic agents (e.g. glucocorticoids and chemotherapy) by transducing signals to bone cells at the surface. Osteocyte-produced FGF-23 acts on kidneys to regulate phosphate and calcium homeostasis (Bergwitz and Juppner, 2010), and can initiate targeted remodeling (Heino et al., 2009).

Osteoclasts (OCs) are the only cell type responsible for bone resorption. They are multinucleated giant cells derived from hematopoietic stem cells of the monocyte/macrophage lineage through a multistep process (osteoclastogenesis)(Boyle et al., 2003, Väänänen and Laitala-Leinonen, 2008)(**Figure 2.3**). Osteoclastogenesis involves many cytokines and hormones but is mainly dependent on OB-produced M-CSF and RANKL, and osteocyte produced sclerostin. RANKL is the key regulator, important also for mature OC activation. Activation of RANK is inhibited by OPG, also produced by OBs. The RANKL/OPG ratio regulates RANK activation. Osteotrophic factors including PTH, vitamin D, interleukin (IL) 11 and PEG_2 stimulate OB production of RANKL and inhibit OPG production. Increased bone resorption in postmenopausal osteoporosis and other skeletal disorders is associated with increased production of RANKL in proportion to OPG (Manolagas, 2000). The lifespan of OCs is 2 weeks (Boyle et al., 2003, Väänänen and Laitala-Leinonen, 2008).

Osteal macrophages (osteomacs) is a new class of bone cells identified in the bone remodeling compartment, functioning as regulators of bone formation (Chang et al., 2008, Pettit et al., 2008). Resident tissue macrophages are part of immune responses and central for tissue homeostasis. Osteomacs represent a distinct bone population of resident tissue macrophages (Chang et al., 2008, Alexander et al., 2011). They are identified by expression of surface marker F4/80 and location on or near to periosteal and endosteal bone surfaces. Osteomacs participate in bone modeling by forming a canopy over bone forming OBs (Chang et al., 2008, Pettit et al., 2008), suggesting a role as coupling-factor (**Figure 2.3**).

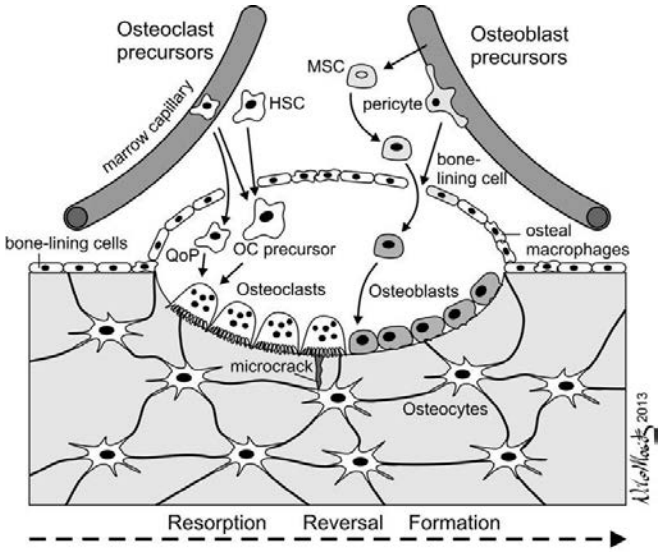

FIGURE 2.3 ***Bone cells, their recruitment and actions during the four phases of bone remodeling by the multicellular unit (BMU) in the bone remodeling compartment (BMU).*** *In the activation phase, a quiescent bone surface is transformed into a remodeling surface (left part). Osteoclast (OC) precursors are recruited, differentiated and activated. Bone lining cells and osteomacs create a closed bone remodeling compartment (BRC) where the required biochemical microenvironment is created. The BRC is in close proximity to the vasculature providing cells through capillaries penetrating the canopy. During the resorption phase, bone matrix is removed by osteoclasts In the reversal phase coupling mechanisms are responsible for the switch between resorption and formation. Resorption pits are populated by monocytes, MSCs and preosteoblasts. The resorbed bone surface is prepared for formation, most likely by MSCs and/or bone lining cells, producing a thin layer of collagen for osteoblast attachment. Thereafter follows bone formation with subsequent mineralization. Figure modified from Sims and Walsh 2012, Khosla et al., 2010, Baron and Kneissel 2013, Martin et al., 2013. Illustration by Niko Moritz.*

2.2.3 Bone remodeling and repair

Bone formation can take place through three different processes; indirect bone formation through a cartilage template – *endochondral ossification*, direct formation of an organic matrix without a pre-existing template – *intramembranous ossification*, or by synthesis of bone matrix on the surface of existing bone tissue – *appositional ossification*. In postnatal life, bones grow in length by endochondral ossification and in width by intramembranous ossification and periosteal apposition. Bone tissue is continuously renewed through remodeling, a lifelong turnover process replacing dead or injured bone tissue, enabling the skeleton to adapt to mechanical, nutritional and/or metabolic changes (adaptive remodeling) and to repair itself without scar tissue formation. The functional adaption to mechanical stress is referred to as Wolff's law, as presented by the anatomist Julius Wolff in 1892 (Wolff 1892). Increased loading of a bone result in increased bone strength, while decrease or lack of mechanical load leads to decreased bone density and reduced strength. Mechanical loading stimulates continuous remodeling through activation of cellular and biochemical responses ultimately responsible for changes in the bone architecture (Frost, 1994, Huiskes et al., 2000). As mechano-sensors of bone tissue, osteocytes are integral for initiation of this mechanotransduction pathway. These processes are observed in osseointegration of cementless hip implants.

Bone remodeling

Bone remodeling, i.e. turnover of bone tissue where old or damaged bone is removed by osteoclasts and replaced by osteoblasts, is the most central skeletal process through which bone exerts its physiological functions. The process is driven by the basic multicellular unit (BMU). The four distinct phases of the bone remodeling cycle are: activation, resorption, reversal and formation (**Figure 2.3**). The interplay between bone cells, matrix proteins and external factors are of outmost importance in this well balanced process where also T- and B-cells and neural cells seem to be involved (Kular et al., 2012). In adult bone, resorption in a BMU lasts for approximately 3 weeks, while formation takes 3-4 months. Remodeling is simultaneously taking place at multiple sites throughout the skeleton in an asynchronized way. Since remodeling is a surface process, the remodeling rate of trabecular bone is much faster. In normal healthy adults the remodeling rate of cortical bone is estimated at 2-5% per year, and 5-10 times faster in trabecular bone. On average, 5-10% of the skeleton is replaced annually, and the entire skeleton in 10 years (Parfitt, 1982, Martin et al., 2013). Bone remodeling is tightly coupled and regulated to ensure proper progress through the remodeling cycle. To maintain bone mass the exact amount of resorbed bone has to be replaced during formation. Disruption or imbalance can result in a number of bone diseases (e.g. OP, osteopetrosis or Paget's disease). Normal aging, hormonal changes, nutritional factors and decreased physical activity cause imbalanced remodeling, resulting in bone loss and decreased bone quality.

Repair

Due to the natural turnover process the skeleton has a unique ability to self-repair through regeneration of the original tissue with all its morphological, structural and functional properties (Buckwalter et al., 1996). Demographic factors including age, bone quality and medical disorders affect the healing mechanisms and outcome (Borrelli et al., 2012). Fracture healing is the best described form of bone healing (Einhorn, 1998, Gerstenfeld et al., 2003, Aro et al., 1990), providing a point of reference for description of the process at the tissue, cellular, molecular and genetic levels. Under optimal conditions, healing occurs through intramembranous ossification, utilizing the normal remodeling system (Day et al., 2000). In reality, some degree of micromotion always takes place at the healing site and bone healing takes place through a combination of endochondral and intramembranous ossification (Einhorn, 1998, Aro and Chao, 1993). As in remodeling, the process is dependent on the vasculature to provide MSCs, perivascular stem cells and preosteoblasts to the healing site (Khosla et al., 2010). Osseointegration of bone implants is one form of bone healing, taking place through intramembranous ossification (Goodman et al., 2009).

2.2.4 Osteogenic differentiation and bone matrix formation

Osteoblasts are primarily derived from MSCs that are thought to migrate from the bone marrow and undergo stepwise differentiation. Osteoblast lineage development includes transition stages from MSC to osteoprogenitor, preosteoblast, and mature osteoblast. The complex differentiation process is characterized by timely expression of transcription factors and osteoblastic genes followed by extracellular matrix synthesis and subsequent mineralization.

Most of what is known about osteoblast lineage cells and the regulation of their differentiation, survival and function derives from studies on cultured cells. Compared to early studies on rodent OBs, osteosarcoma or OB cell lines, the discovery of MSCs has revolutionized *in vitro* studies on differentiation. Osteogenic differentiation of MSCs can be divided into three stages; 1) proliferation, 2) differentiation, extracellular matrix (osteoid) development and maturation, and 3) mineralization (Aubin, 2008). Each step is associated with characteristic changes in gene and protein expression (**Figures 2.4**).

TRANSCRIPTION FACTORS

Transition from MSC to mature osteoblast requires activity of specific transcription factors (TFs) at distinct time points defining various stages of differentiation (Aubin, 2008). Three major TFs determine development from MSCs to preOBs and further to mature osteoid producing osteoblasts: SOX9, RUNX2 and osterix.

SOX9 defines the transition from MSC into a common chondro-osteogenic lineage and is crucial for chondrogenesis (Akiyama et al., 2005), but its role in osteogenesis is less understood. SOX9 is not expressed by mature osteoblasts (Long, 2012).

RUNX2 (runt family transcription factor 2, formerly Cbfa1) is the main regulator of osteoblast-lineage cells. It is indispensable for osteogenic differentiation, as demonstrated by lack of OBs and bone tissue in RUNX2 knockout mice (Otto et al., 1997). RUNX2 is the earliest marker of osteogenic differentiation, but is also required in mature OBs for proper function and matrix synthesis. Various nuclear factors are known to interact with RUNX2 to promote differentiation by stimulating its expression, enhancing its activity, or acting as coactivators. Additional factors suppress RUNX2 (Long, 2012).

Osterix (OSX) is a bone morphogenetic protein (BMP)-induced TF, functioning downstream of RUNX2. Deletion of the OSX gene leads to lack of OBs and ectopic cartilage formation (Nakashima et al., 2002). OSX is crucial for osteogenic and chondrogenic differentiation both during development and in postnatal bone remodeling (Zhou et al., 2010b). Many other TFs regulate osteogenesis by regulating OSX, including tumor suppressor p53 and the Ca-sensitive transcription factor NFATC1 (nuclear factor of activated T cells, cytoplasmic 1) that suppresses and stimulates OSX activity, respectively (Long, 2012).

The activating transcription factor 4 (ATF4) is a regulator of mature OBs, affecting the expression of OCN and RANKL.

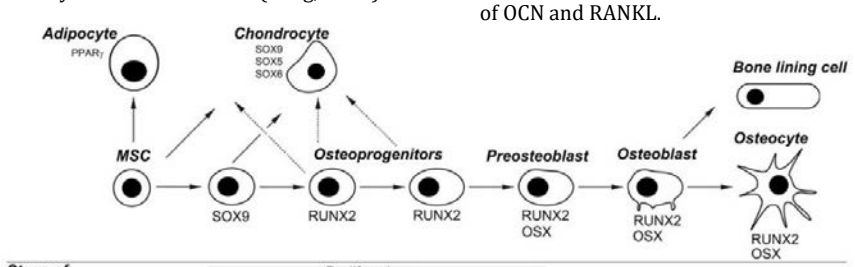

FIGURE 2.4 *Overview of osteoblast lineage differentiation of MSCs with the stage-characteristic changes in proliferative activity and osteogenic gene expression indicated. OSX, osterix; ALP, alkaline phosphatase; Col, collagen; OPN, osteopontin; BSP, bone sialo protein; OCN, osteocalcin. (Modified and adopted from Aubin, 2008 and Long, 2012).*

SIGNALING PATHWAYS IN OSTEOGENIC DIFFERENTAITION

Several developmental signaling pathways regulate TFs and ensure proper progress through the differentiation stages. These include Hedgehog, Notch, Wnt, BMP and FGF signaling pathways (Long, 2012). The Wnt/β-catenin pathway (canonical Wnt) has been considered the most important in regulating osteogenic differentiation (De Boer et al., 2004, Baron and Kneissel, 2013)(**Box 2**), but the non-canonical pathway is also crucial (Lerner and Ohlsson, 2015).

BOX 2. The canonical Wnt signaling pathway

Canonical Wnt signaling is initiated when activated Wnt binds to its membrane receptor complex, composed of Frizzled (FZD) G protein-coupled receptors and a low-density lipoprotein receptor-related protein (LRP), LRP5 or LRP6. This leads to inactivation of the cytoplasmic multiprotein β-catenin destruction complex, releasing the signaling mediator β-catenin. Accumulated β-catenin translocate into the nucleus and associates with TFs to regulate transcription of target genes. Wnt/β-catenin signaling regulates osteogenesis through multiple mechanisms and is essential for controlling bone homeostasis. Wnt activation is required for OB lineage commitment and promotes OB differentiation indirectly by suppressing adipogenic TFs including PPARγ, and promoting osteogenic over chondrogenic differentiation. Wnt promotes proliferation of MSCs, osteoprogenitors and mature OBs, and stimulates mineralization, while reducing apoptosis of mature OBs. Wnt induced OB expression of OPG contribute to reduced osteoclastogenesis. The Wnt signaling system interacts with several pathways to regulate bone mass, including PTH and BMP signaling. (reviewed in Baron and Kneissel 2013, Lerner and Ohlsson 2015)

Recent data indicate that the key mediator of embryonic stem cell maintenance, Nanog, may be involved in MSC maintenance, differentiation and bone remodeling (Bais et al., 2012). The signaling pathways are post-transcriptionally regulated by a range of microRNAs, as previously demonstrated by us (Laine et al., 2012) and others and extensively reviewed by Lian et al., (2012). A panel of miRNAs with stage-specific roles in osteoblastic and osteocytic differentiation has been identified, designated as OstemiR (Eguchi et al., 2013). Due to their important regulatory roles, miRNAs are suggested targets for enhancing OB differentiation and bone formation in future therapeutic applications (Taipaleenmäki et al., 2012).

GENE EXPRESSION PATTERN, BONE FORMATION AND MINERALIZATION

Expression of OB-specific genes are up- or downregulated asynchronously as the differentiation process proceeds (**Figure 2.4**). Based on expression patterns of 12 essential OB associated genes, osteoblastic cells undergo seven transition stages during differentiation (Liu et al., 2003). The early stages are characterized by proliferation of MSCs, progenitors and preOBs, and associated with expression of RUNX2, histones, proto-oncogenes (c-fos, c-myc), cyclins (B and E), fibronectin, osteopontin, bone sialoprotein and TGF- β. By the end of the proliferative stage, transcription of OB-specific genes is upregulated (except osteocalcin), indicating that differentiation start before progenitors leave the proliferative stage (Aubin, 2008).

Next follows differentiation into preOBs, characterized by strong ALP and COL1 expression, while expression of osteopontin and bone sialoprotein decreases transiently (Aubin, 2008). OSX expression is initiated, while RUNX2 is continuously expressed. OBs start to lay down collagen matrix at an early stage. Consistent with increased matrix synthesis, expression of matrix proteins and ALP peaks during maturation from preOBs to OBs. The NCPs embedded into the collagen matrix bind calcium for incorporation onto the growing mineral crystals (Robey and Boskey, 2008).

During mineralization, expression of ALP decreases and OCN appears. The basic mechanism for biological HA deposition is heterogeneous nucleation (Glimcher, 1987), where organic or inorganic precursors guide the formation of solid apatite from soluble calcium and phosphate ions (Termine, 1972, Golub, 2011). Initiation of mineralization requires three critical constituents: 1) presence of a collagen network, 2) local increase in precipitating ion concentrations, primarily phosphate, and 3) removal or modification of mineralization inhibitors (Murshed and McKee, 2010). Extracellular concentrations of phosphate and calcium ions are highly critical (Golub, 2011). ALP is the key modulator in mineralization (Lorch, 1949), generating phosphate ions (Pi) by hydrolyzing inorganic pyrophosphate (PPi) (Allori et al., 2008b). Osteonectin, bone sialoprotein and phosphoproteins are suggested promoters of mineralization, whereas OCN and proteoglycans are among suggested regulators. Osteopontin, PPi, and large proteoglycans are inhibitors (Robey and Boskey, 2008, Murshed and McKee, 2010).

Late osteoblastic stage and osteocytes are associated with DMP1 expression. Cells undergo transition into osteoblastic osteocytes and osteocytes, expressing sclerostin which inhibits BMP and Wnt signaling, thereby reducing the number of MSCs entering the differentiation process (Aubin, 2008, Lian et al., 2012).

FACTORS AFFECTING OSTEOGENIC DIFFERENTIATION AND BONE FORMATION

Osteogenic differentiation, maturation, maintenance and bone formation is regulated by a range of local and systemic signaling molecules, including:

- Growth factors: FGFs, insulin-like growth factor 1 (IGF-1), platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β) and their receptors
- Hormones (i.e., PTH, leptin, sex hormones)
- Cytokines: IL-1, -6, and -11, tumor necrosis factor α (TNF-α) and leukemia inhibitory factor (LIF)

- Insulin, glucocorticoids, nutritional factors, vitamin D and calcium

Despite well-documented effects on bone formation, overlapping functions of these factors and interactions with signaling pathways are not completely understood. Actions of several factors depend on the differentiation stage, with opposing effects on progenitors and mature cells (Aubin, 2008).

Osteocyte produced *sclerostin* seems to inhibit bone formation through several mechanisms, including binding to several BMPs and thereby inhibiting BMP-mediated differentiation (Kusu et al., 2003). There are also evidences that sclerostin inhibit activation of Wnt-signaling (Baron and Kneissel, 2013). Osteoclast-produced factors affecting OBs and their function include cytokine cardiotropin-1 that stimulate bone formation (Walker et al., 2008) and Semaphorin 4D which inhibits OB-lineage cells and mineralization (Negishi-Koga et al., 2011). Mature OCs recruit MSCs and osteoprogenitors through sphingosine-1-phosphate and BMP-6 production, and stimulate bone formation by activation of Wnt/BMP pathway (Pederson et al., 2008). More recent research has demonstrated the involvement of NF-κβ in bone formation, although mechanisms are poorly understood and reports are contradictory. Collectively preclinical and *in vitro* studies indicate that activation of NF-κβ signaling in mature OBs inhibit bone formation, while inhibition of NF-κβ signaling can increase bone formation and mineralization (Yao et al., 2014). In contrast, NF-κβ seem to promote OB differentiation of MSCs through BMP-2 mediated actions (Hess et al., 2009).

Estrogen is essential in regulating bone remodeling in both genders, and menopause-related estrogen deficiency is associated with bone loss. Both *IGF-1* and *insulin* stimulate OB differentiation and bone formation (Giustina et al., 2008). *Leptin* is considered a critical participant in bone homeostasis, with inhibitory effects on bone formation through mechanisms involving OCN and insulin. However, the exact actions and mechanisms on bone formation are still unclear but appears site-, gender- and time-related (Luther and David 2016).

While high levels of *parathyroid hormone (PTH)* induce bone resorption to release calcium, intermittent levels have positive effects on bone formation (Nissenson and Juppner, 2008). This is facilitated through stimulation of osteogenesis and OB survival, increased MSC recruitment, proliferation and differentiation (Zhou et al., 2011), inhibited adipogenic differentiation, reduced apoptosis, and activation of quiescent bone lining cells (Esbrit and Alcaraz, 2013). The stimulatory effect of PTH on osteoblastic cells is mediated through the PTH/PTHrP receptor type 1 and activation of TFs (Allan et al., 2008). Most effects of PTH seem indirect through other regulatory molecules, i.e. IGF-1 (Yakar et al., 2006) and members of Wnt- (Guo et al., 2010), and BMP-signaling (Yu et al., 2012) pathways.

Vitamin D stimulates OB production of OCN and osteopontin (Prince and Butler, 1987) and regulates OB activity (Howard et al., 1981, Wacker and Holick, 2013). By upregulating RANKL and downregulating OPG in OBs, $1,25(OH)_2D_3$ indirectly stimulate bone resorption and release of calcium and phosphate for reuse in mineralization (Wacker and Holick, 2013). Vitamin D is a major regulator of mineralization. *In vitro* and animal studies have demonstrated additional direct effects of vitamin D on bone. Both $1,25(OH)_2D_3$ (Liu et al., 1999, Zhou et al., 2010a) and the pre-hormonal form $25(OH)D_3$ (Zhou et al., 2010a, Geng et al., 2011) stimulate OB differentiation of MSCs, and $1,25(OH)_2D_3$ seem to regulate RUNX2 (Drissi et al., 2002) and BMP-2 (Piek et al., 2010).

2.2.5 Sources of bone forming cells

Bone marrow is the major source of bone forming cells including MSCs and OB precursors. Osteoblasts and preosteoblasts are found at the endosteal and trabecular bone surfaces within the bone marrow, and in the periosteum. Additional sources are activated in case of bone repair. The periosteum is a well-known source of cells with high bone forming potential. Activation, expansion, and differentiation of periosteal progenitors are essential for initiating neovascularization, bone formation, and remodeling (Colnot et al., 2012), especially in fracture healing (Einhorn, 1998, Colnot et al., 2012).

Under healing conditions, muscle derived stromal cells (MDSCs) can contribute to bone formation (Shah et al., 2013, Liu et al., 2011). Glass and colleagues demonstrated *in vitro* osteogenic differentiation of human MDSCs harvested adjacent to a fracture. Culture supernatants from human fractured tibial bone fragments induced migration and osteogenesis of MDSCs *in vitro*, mediated by low concentrations of TNF-α (Glass et al., 2011).

Animal and clinical studies have demonstrated participation of circulating MSCs in healing of fractures and bone defects. MSCs are not normally detected in the circulation. Upon trauma, MSCs seem to be mobilized through the circulation in response to systemic signals (reviewed in Pignolo and Kassem, 2011). We previously demonstrated circulating MSCs in fracture patients within 2-4 days after fracture (Alm et al., 2010). Under normal conditions, there seem to be a range of circulating osteogenic precursor cell populations (Kuznetsov et al., 2001, Zvaifler et al., 2000, Eghbali-Fatourechi et al., 2005). Indicative of their physiological function, some circulating cell populations have been found elevated during pubertal growth and fracture healing (Eghbali-Fatourechi et al., 2005), and with altered gene expression during postmenopausal bone turnover (Undale et al., 2010).

2.3 MESENCHYMAL STROMAL/STEM CELLS (MSCs)

Mesenchymal stromal/stem cells constitute a heterogeneous subset of stromal precursor cells present at low frequency in mammalian connective tissue compartments. MSCs have been extensively studied for their multiple differentiation and repair capacities, and their immunomodulatory properties. The common MSC concept today is a multipotent cell easy to expand in culture, with regenerative, stromal supportive and immunoregulatory capacities. Still, the true in vivo location and nature of MSCs remains unknown. Bone marrow MSCs is the major source of bone forming cells during normal turnover and in repair and osseointegration of hip implants.

2.3.1 Past and current concepts of MSCs

Mesenchymal stem cells were discovered in the 1970s by Friedenstein and colleagues, as fibroblast colony forming cells (FCFC), found in monolayer cultures explanted from guinea-pig bone marrow and spleen. Cells were isolated by plating whole bone marrow on culture plates and washing away non-adherent cells after a few hours. After 4-5 days, adherent cells started to proliferate and form colonies (Friedenstein et al., 1970). Based on early animal studies on freshly isolated bone marrow cells (Friedenstein et al., 1966, Friedenstein et al., 1968), and *in vitro* cultured FCFCs (Friedenstein et al., 1974), a population of colony-forming unit-fibroblast (CFU-F) was detected, capable of forming bone tissue when transplanted *in vivo* by injection under the renal capsule or in diffusion chambers. These cells were further investigated (Friedenstein et al., 1976, Castro-Malaspina et al., 1980, Mets and Verdonk, 1981, Owen et al., 1987, Caplan, 1991, Haynesworth et al., 1992, Prockop, 1997, Kuznetsov et al., 1997, Bianco et al., 1999, Pittenger et al., 1999, Muraglia et al., 2000) and it was established that plastic adherent, clonal cells from bone marrow were multipotent and could differentiate into OBs, adipocytes and chondrocytes, as well as additional cells of the mesenchymal lineage. Parallel observations revealed that stromal cells in the bone marrow are required for haematopoiesis (Dexter et al., 1977), and constitute key players in the haematopoietic stem cell (HSC) niche (Schofield, 1978, Friedenstein et al., 1968, Friedenstein et al., 1982). The osteogenic properties of bone marrow stromal cells defined them as mesenchymal cells, without defining a stem cell origin.

The bone marrow harbors two overlapping niches for haematopoiesis; the endosteal and the (peri)vascular niche (Adams and Scadden, 2006). In the endosteal niche, the stromal compartment constitute MSCs differentiated to osteoblasts (Long et al., 1990, Adams and Scadden, 2006), whereas the (peri)vascular niche constitute MSCs differentiated into vascular smooth muscle cells (Galmiche et al., 1993, Charbord, 2010). In addition to the supportive role of MSCs in the HSC niche, direct regulatory roles have also been demonstrated (Mendez-Ferrer et al., 2010, Omatsu et al., 2010). MSCs release trophic factors in response to injury. In addition to their regenerative and stromal supportive properties, MSCs also have well documented immunoregulatory properties (Le Blanc et al., 2004, Horwitz et al., 1999, Uccelli et al., 2008, English, 2013, Le Blanc and Mougiakakos, 2012).

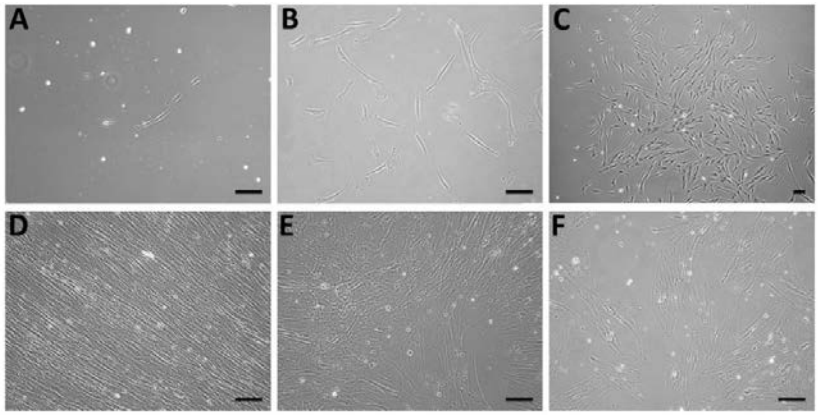

FIGURE 2.5 ***Bone marrow MSCs.*** *(A) Adherent cells visible 2 days after seeding bone marrow MNCs, with a few cells of MSC morphology. (B) After additional days in primary culture increasing numbers of MSCs are visible in emerging colonies referred to as colony forming units (CFUs). (C) After 10-15 days in primary culture larger colonies of cells with typical MSC morphology can be detected. (D) Passaged cells reach confluence within 7-21 days (depending on the proliferative capacity of individual cells and culture conditions). (E-F) With increased passaging and expansion MSCs lose their spindle-shaped morphology and gradually become wider and flattened as they approach senescence. Scale bar=100*

2.3.2 Isolation and culture expansion

ISOLATION

MSCs can be found in a range of tissues, but bone marrow, adipose tissue and umbilical cord are the most commonly used sources, mainly due to higher frequency of MSCs, ease of harvest and availability of tissue. In spite of extensive research on MSCs for over 40 years, the original isolation protocol is still in use. Bone marrow MSCs are readily isolated by plating the low-density mononuclear cell fraction isolated by density gradient centrifugation, or whole marrow, in culture dishes, as recently reviewed elsewhere (Alm et al., 2014). MSCs are isolated from other cell types through their adherence to culture plastic and ability to proliferate through several passages.

CHARACTERIZATION

In primary culture, MSCs form colonies (CFU-Fs) of proliferating cells with a spindle-shaped, fibroblast-like morphology (**Figure 2.5**). The main characteristics of MSCs are their ability to continue to proliferate through several passages in culture, and trilineage differentiation into osteoblasts, adipocytes and chondrocytes (Pittenger et al., 1999). The isolated MSC populations are heterogeneous with coexisting subsets of cells with differing phenotype and differentiation potential. Some of the single-cell derived colonies display multipotency, whereas other subpopulations express more restricted potential (Kuznetsov et al., 1997, Pittenger et al., 1999, Phinney, 2007).

Extensive attempts have been made to enrich cultures for multipotent MSCs and to identify a specific surface marker or marker profile. MSCs are known to express a broad range of non-specific markers, and although vigorously investigated no unique MSC marker has yet been found. It is generally agreed that MSCs do not express the hematopoietic markers CD34, CD45, CD14 or CD11, or the adhesion molecules CD31, CD18 or CD56. They can however express CD105, CD73, CD90, CD71, CD271, and adhesion molecules CD106, CD166, CD54 and CD29, among others (reviewed in Boxall and Jones 2012). In an effort to overcome the heterogeneity in the field, ISCT proposed minimal phenotypic and functional criteria for identifying MSCs (Dominici et al., 2006)(**Box 3**).

BOX 3. ISCT Minimal criteria for identifying MSCs
(Dominici et al., 2006)

- Adherence to plastic in standard culture conditions
- *In vitro* differentiation into osteoblasts, adipocytes and chondrocytes
- Expression of CD105, CD73 and CD90
- Lack of expression of:
  - monocyte and macrophage markers (CD11b or CD14)
  - a haematopoietic progenitor and endothelial marker (CD34)
  - a leukocyte marker (CD45)
  - B-cell markers (CDCD19 or CD79a)
  - HLA-DR

So far, there is no clear strategy available to distinguish multipotent MSCs from other precursor cells and phenotype analyses are mainly applied for characterization of culture expanded cells. The patterns of phenotypic markers are altered in culture and the phenotype of freshly sorted bone marrow MSCs can be one of several (Jones et al., 2002, Qian et al., 2012, Tormin et al., 2011). Collectively, studies indicate that a $CD271^+/CD146^+/CD44^-$ phenotype on freshly isolated non-hematopoietic bone marrow cells can represent an immature and potent subset of MSCs (Sacchetti et al., 2007, Tormin et al., 2011, Qian et al., 2012). CFU-Fs are considered primary MSCs.

MSCs from different tissues may display biological and phenotypic differences but share common features. Tormin and colleagues (2011) demonstrated that CD146 expression on primary human MSCs correlate with the *in situ* localization within the bone marrow. Taken together, data indicate that MSCs are a product of their environment, *in vivo* and *in vitro*, and their ability to adapt to changes in their environment is part of their true nature. Due to the low frequency of MSCs *in vivo* and the lack of a known unique phenotype, research relies so far still on culture expanded cells.

CULTURE EXPANSION

Growth kinetics of cultured MSCs is of interest due to the need of optimal expansion conditions to obtain sufficient cell numbers for research and therapeutic applications (reviewed in Alm et al., 2014). MSCs have high proliferative capacity *in vitro*, but as any human somatic cell type culture expansion is limited due to cellular senescence. The number of population doublings (PDs) reached before entering senescence and ultimately stoped proliferation, the Hayflick limit (Hayflick, 1965), varies for different cell types. The collective data suggest that adult MSCs have a Hayflick limit of approximately 50 PDs.

In culture, MSCs show an initial phase of high proliferation rate, followed by a plateau of slower proliferation and eventually cessation of proliferative activity (Bruder et al., 1997, Banfi et al., 2000). With increased passaging and expansion, MSCs lose their spindle-shaped morphology and gradually become wider and flattened as they approach senescence. Long-term growth kinetics vary and reported maximum PDs for adult MSCs range between 4-25 (Digirolamo et al., 1999, Wagner et al., 2008), 30-40 (Bruder et al., 1997, Banfi et al., 2000), or 50-60 (Stenderup et al., 2003, Charbord, 2010).

The genetic stability is retained in human MSCs through culturing, but after passage 4-5 random chromosome alterations can be detected. Normal karyotype can be retain up to passage 20 (Tarte et al., 2010, Binato et al., 2013). Despite reduced proliferation, karyotypic aberrations and loss of

multipotency with increased passaging (Banfi et al., 2000, Satija et al., 2007), the osteogenic and adipogenic differentiation capacity seem retained through extended culturing (Binato et al., 2013).

Optimal culture conditions often involve pre-selected FCS batches for supporting proliferation. Human MSCs expand more rapidly and retain their MSC profile if seeded at low density. Comparing densities from 1,000 cells/cm^2 to 200,000 cells/cm^2 in primary culture, and 50 to 1,000 cells/cm^2 for passaged cells, increasing cell yield have been demonstrated with decreasing density (Sotiropoulou et al., 2006, Colter et al., 2000, Sekiya et al., 2002). Still, densities ranging between 1,000 and 4,000 cells/cm^2 are most commonly applied for expansion.

MSC THERAPY

The osteogenic potential of MSCs *in vitro* has brought extensive hope and research interest within the field of bone tissue engineering for development of cell-based treatment options (Service, 2000, Muschler et al., 2004, Griffin et al., 2011). Combining autologous MSCs with bioscaffolds, such as hydroxyapatite, has proven functional for repair of segmental bone defects in numerous animal models (Ohgushi et al., 1989, Gao et al., 2012), and in proof-of-concept clinical studies (Quarto et al., 2001). However, the clinical use of MSC in orthopaedic settings remains challenging and slow. MSC therapy is used in a broad spectrum of clinical non-orthopaedic applications, most commonly as immunosuppressors in graft versus host disorders and autoimmune disorders (Martin et al., 2016). There are over 600 clinical trials with human MSCs registered at the moment (www.clinicaltrials.gov, accessed April 2016), of which only a minor part is related to bone regeneration. Clinical studies on MSC therapies in orthopaedics and skeletal trauma are so far mostly observational. Bone marrow or *ex vivo* expanded MSCs have been used in adult fracture patients to treat non-unions or delayed unions, for healing of bone defects, or as treatment for avascular osteonecrosis. These studies have established the feasibility and safety of MSC therapy in orthopaedic applications, but efficiency of such therapy has to be confirmed (Gomez-Barrena et al., 2011). Another promising application of MSCs in orthopaedics is for impaction of bone graft to improve regeneration of bone stock in revision THA (Bolland et al., 2007, Korda et al., 2010).

2.3.3 *In vitro* OB differentiation of MSCs

The *in vitro* osteogenic assay serves three main research purposes: 1) as part of minimal criteria for defining MSCs, 2) as an invaluable culture model in bone research, and in testing osteogenic response to drugs, hormones and implant materials, and 3) as a fundamental part of bone tissue engineering.

Culture conditions

The classical and well established protocol for osteoblastic differentiation of human MSCs *in vitro* is culturing sub-confluent MSCs in presence of ascorbic acid (AA), β-glycerophosphate (βGP) and dexamethasone (Dex). Additional osteogenic factors, such as vitamin D and BMPs, have also been used successfully. There is a plethora of studies from the last 30 years testing a range of molecules for their ability to accelerate or influence the *in vitro* osteogenic process. Still, the basic protocol has remained relatively unchanged. Over recent years, several ready-to-use commercial induction media have been developed providing a convenient solution, especially if the purpose is solely to demonstrate osteogenic capacity as part of characterizing MSCs. However, the composition and concentrations of crucial inductive agents are not always described. If the differentiation process itself is the subject for investigation, or the aim is to produce fully mature and functional matrix producing OBs, the composition of inductive agents has to be defined, since there might be need for fine tuning concentrations, timing and duration of supplements.

Ascorbic acid is essential for collagen matrix maturation. As a cofactor for hydroxylation of abundant proline and lysine residues in collagens, AA is required for proper collagen fibril assembly (Pinnel et al., 1987, Choi et al., 2008). AA also increases synthesis of non-collagenous bone matrix proteins and total protein. Supplementation of human MSCs with AA upregulate cell cycle and mitosis related genes (Fernandes et al., 2009), and stimulates proliferation. In mature osteoblasts, AA stimulates survival and maintenance of osteoblastic phenotype (Beresford et al., 1993, Jaiswal et al., 1997, Choi et al., 2008). In absence of Dex and β-GP, AA have little impact on ALP expression (Jaiswal et al., 1997), but absence of AA reduce ALP expression and inhibit calcium accumulation (Fernandes et al., 2009). For cell culture, the more stable form ascorbic acid 2-phosphate is used, usually at concentrations ranging from 50 to 500 μM (Gupta et al., 2011, Langenbach and Handschel, 2013).

β-glycerophosphate is used as an organic phosphate source for *in vitro* bone formation. βGP is enzymatically hydrolysed into inorganic phosphate by ALP (Beresford et al., 1993, Langenbach and Handschel, 2013). As a consequence, increasing βGP concentrations does not increase mineralization, since the ratio between PPi and Pi is the critical factor (Hessle et al., 2002), fully dependent on ALP activity (Allori et al., 2008b). Concentrations between 5 and 10 mM are normally used (Gupta et al., 2011).

Seeding density has impact on the osteogenic assay. In general, MSC differentiation is triggered by increasing cell density. Mineralization of *in vitro* assayed MSCs increases with seeding density up to a

certain threshold of approximately 5,000 to 7,500 cells/cm^2. Higher densities are associated with cell detachment and increased adipogenesis (Jaiswal et al., 1997, Aubin, 2008). Confluence of 60-70% is known to generate more consistent results. Seeding densities ranging between 1,000 and 10,000 cells/cm^2 are generally used (Krause et al., 2011, Gupta et al., 2011). Accumulation of soluble factors produced by MSCs during osteogenic induction promote differentiation and mineralization (Jaiswal et al., 1997), therefore changing of only half the media volume can further promote bone formation *in vitro*.

The *in vitro* differentiation process

In vitro differentiation and bone formation follows the events outlined above. The proliferative stage last for approximately 10 days, producing the required numbers of cells, followed by increasing expression of ALP, production of a collagen matrix, and subsequent expression of OCN and osteopontin, followed by calcium and phosphate deposition (Aubin, 2008). The fully differentiated osteoid producing OB is characterized by a cuboidal morphology and a strong ALP expression (**Figure 2.6**). As the differentiation process proceeds, three dimensional structures (nodules or strings) start to form which are mineralized. Depending on the experimental settings and donor characteristics, the differentiation stage start at day 7 to 10 and can last between 10 to 21 days, while mineralization is detected after 3 to 5 weeks.

In vitro OB differentiation of MSCs is associated with large variations reflecting differences in starting material (tissue source, phenotype, subpopulation selection), culture conditions (plating density, supplements), donor related factors, and sensitivity of read-out methods (Jaiswal et al., 1997, Aubin, 2008). A main cause of varying results is the heterogeneity within any MSC population, independent of tissue source and donor profile (De Bari et al., 2010). Some subpopulations undergo spontaneous differentiation while other require stimuli, and heterogeneity in gene expression and functional outcome can reflect different osteogenic stages (Aubin, 2008, Rothenberg et al., 2011).

Detection methods

Osteogenic differentiation is generally detected by measurement of ALP activity and visualized by ALP staining. Mineralization is detected by staining for phosphate (von Kossa staining) (**Figure 2.6**) and calcium (Alizarin Red) deposition. Osteogenic commitment can be evaluated by analyzing expression of early (RUNX2, OSX), intermediate (ALP, COL1, osteopontin) and late-stage (osteonectin) osteogenic genes. While ALP is a measure of early OB differentiation, mineralization is a late marker of complete differentiation into mature, functional, matrix producing OBs (Krause et al., 2011, Gupta et al., 2011). Mineralization is more difficult to achieve *in vitro*, and results are more unreliable due to assay related limitations affecting the outcome. This is probably the reason why mineralization is not always reported when osteogenic capacity of human MSCs is investigated. Quantification of bone formation markers at the protein level could provide useful functional information, but assays are expensive and not always optimal for cell culture applications and are therefore of limited use.

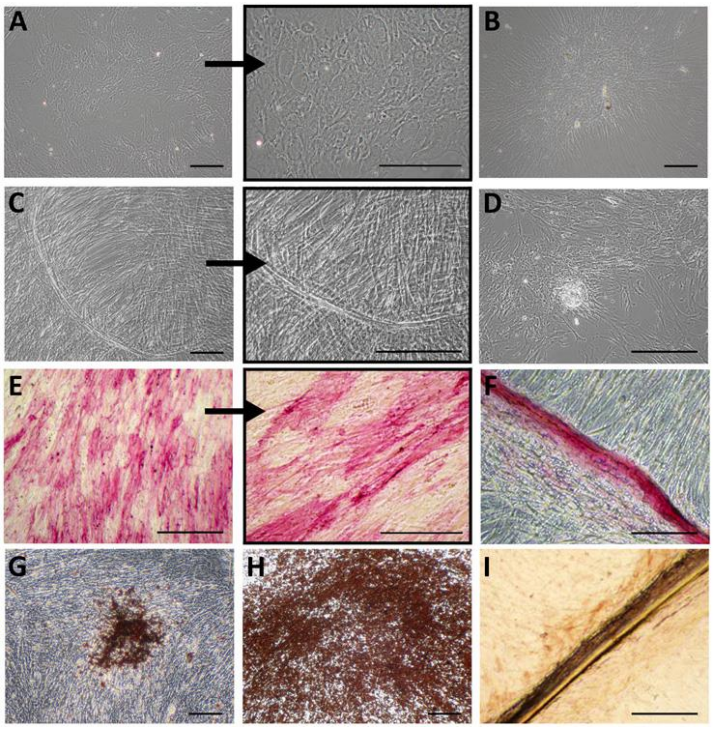

FIGURE 2.6 ***In vitro osteogenic differentiation of human MSCs.*** *As the MSCs differentiate into osteoblasts they attain a cuboidal morphology (A) and start to form three dimensional structures (B-D). Differentiation can be detected by ALP staining (E-F) starting from day 7-14 (depending on individual variations). After 3-5 weeks bone matrix mineralization can be detected by von Kossa staining (G-I) as immature brownish osteoid or darker brown mature bone matrix. Scale bars=100 µm.*

2.3.4 Dexamethasone as an *in vitro* osteogenic agent

Dexamethasone is a potent modulator of *in vitro* osteogenic differentiation of MSCs, and was introduced as a supplement based on the involvement of endogenous systemic glucocorticoids (GCs) in *in vivo* bone formation and remodeling (Maniatopoulos et al., 1988, Leboy et al., 1991). As other GCs, Dex has complex effects on MSCs and osteoblastic cells. Compatible with the clinically negative side effects of high and prolonged levels of endogenous or synthetic GCs leading to bone loss (Canalis, 2005), *in vitro* effects of Dex on osteogenic cells is not solely beneficial and conflicting results exists (**Table 2.2**). Based on dose responses studies with ranges of 10 pM to 1 000 nM, concentrations corresponding to physiological GC levels, i.e., 10 to 100 nM, have been established to provide maximal stimulatory effect on human MSCs (**Table 2.2**). While most studies demonstrate that Dex is required for mineralization, a few reports show complete OB differentiation and mineralization also without Dex (**Table 2.2**). For human MSCs, both 10 and 100 nM Dex are commonly used concentrations (Vater et al., 2011, Krause et al., 2011).

The underlying biological mechanisms and pathways by which Dex affects OB lineage cells are still poorly understood. Whether Dex inhibits or promotes OB differentiation and bone formation of MSCs depends on the experimental set-up. Responses are largely dependent on the differentiation stage of the cells and the dose and duration of Dex supplementation (**Table 2.2**). Primary cultures of MSCs tend to contain a heterogeneous population of cells at different commitment stages. This can explain the differences in responses observed in primary and passaged MSCs. In addition, the osteogenic response to Dex may vary with age (D'Ippolito et al., 1999, Shur et al., 2005) and skeletal health status (Pei et al., 2003, Rao et al., 2005, Zhou et al., 2012) of the donor, explaining discrepancies in the literature. It is clear that human and animal cells respond differently to Dex (Beresford et al., 1994, Cheng et al., 1994, Ito et al., 2007), accounting for further inconsistencies in literature.

MOLECULAR MECHANISMS OF DEX ACTIONS

Dex has been recognized involved in BMP-2 and BMP-6 responses in human MSCs (Jager et al., 2008, Liu et al., 2004, Jorgensen et al., 2004) and to mediate effects of vitamin D (Beresford et al., 1994, Fromigue et al., 1997, Jaiswal et al., 1997) by increasing VDR transcription (Hidalgo et al., 2011). The detailed mechanisms for Dex mediated transcriptional effects on OB differentiation and bone formation have been poorly understood. Based on recent investigations, molecular pathways and target molecules have becoming revealed, indicating effects through both BMP- and Wnt-signaling pathways.

Extracellular GCs and Dex can reach their intracellular targets through several routs. In skeletal cells, these mechanisms are not always distinguishable (Hong et al., 2008) (**Figure 2.7A**). In the cytoplasm, inactive GCs are converted into active forms by the enzyme 11β-HSD1. This enzyme is expressed in a range of tissues, including skeletal cells, and is essential in osteogenic differentiation and bone formation. In differentiating OBs, mRNA expression of 11β-HSD1 is low but persistent in presence of Dex, and increase in absence of Dex (Eijken et al., 2005), indicating 11β-HSD1 expression as a feedback mechanism to increase availability of active GC in absence of Dex (Eijken et al., 2006). The isoenzyme 11β-HSD2 has opposite actions, converting active cortisol GCs into inactive cortisone (Hong et al., 2008)(**Figure 2.7A**). Human OBs express 11β-HSD2, but the level is much lower than 11β-HSD1 (Cooper et al., 2000). Increased 11β-HSD2 activity reduces availability of active GCs, causing decreased GC signaling and reduced osteogenic responses (Hong et al., 2008).

Dex exerts osteogenic effects on MSCs through several mechanisms that induces and regulates RUNX2. Dex induces RUNX2 expression through the transcriptional modulator FHL2 (four and a half LIM-only protein) mediated Wnt/β-catenin-dependent

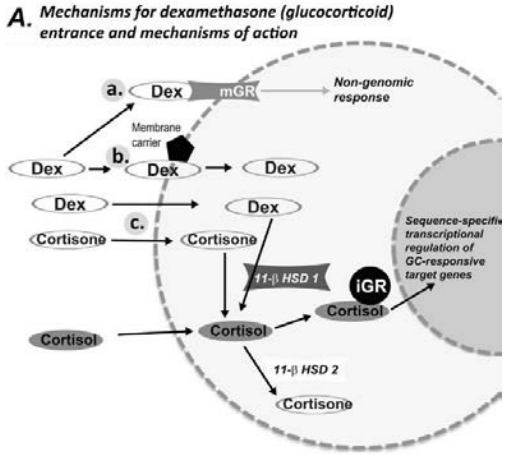

FIGURE 2.7A *Dex can enter the cell through binding to a membrane GC receptor (mGR) to trigger rapid non-genomic response (a), through membrane carrier-mediated uptake (b) or pass across the plasma membrane through simple non-specific diffusion (c). In the cytoplasm, inactive Dex/GCs are converted into active forms by the enzyme 11ß-HSD1. Cortisol and other active forms of GCs bind with high affinity to the intracellular GC receptor (iGR), which is then released from its inactive complex and translocates into the nucleus where it serves as a DNA sequence-specific transcriptional regulator of distinct GC target genes. Modified from Hong et al 2008.*

transcriptional activation (Hamidouche et al., 2008), enhances RUNX2 activity by upregulation of TAZ (transcriptional co-activator with PDZ-binding motif)(Hong et al., 2008, Hong et al., 2009)(**Figure 2.7B**), and can regulate RUNX2 through activation of MKP-1 (mitogen-activated protein kinase phosphatase 1)(Phillips et al., 2006). Increasing evidences suggest that FHL2 is a crucial factor for osteogenic commitment of MSCs, and the key mechanism through which Dex induces osteogenic differentiation in MSCs is through upregulation of FHL2 (Langenbach and Handschel, 2013).

TRANSIENT DEX TREATMENT

Complete osteoblastic maturation and mineralization has been suggested to require continuous Dex treatment for 21-28 days. However, long-term Dex treatment is also associated with decreased viability, especially with 100 nM Dex (**Table 2.2**). Cheng and colleagues (1994) investigated transient 100 nM Dex treatment of passaged MSCs and found ALP activity and mineralization comparable to continuously treated cultures. Supporting a sustained effect after withdrawal, 48 h Dex (10 nM) treatment of primary MSCs demonstrated improved adherence, proliferation and ALP expression, with continued increased ALP and COL1 expression in secondary culture (Fromigue et al., 1997). In a similar study, 48 h Dex treatment of primary cultures resulted in increased proliferation, ALP activity and mineralization after secondary culture without Dex (Schecroun and Delloye, 2003). Song and co-workers (2009b) reported increased ALP activity with increased treatment time using 100 nM Dex, but it is unclear whether their study was performed on primary or passaged cells. Commitment to an end-stage phenotype is determined early in differentiation culturing (Jaiswal et al., 1997), and results collectively indicate that short-term Dex treatment is sufficient to promote complete osteogenic differentiation of human MSCs. Still, this is not the established protocol.

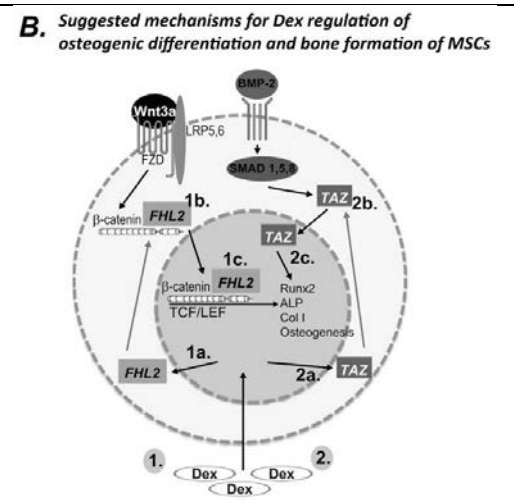

FIGURE 2.7B *One suggested mechanism of action for Dex is through interactions with Wnt-signaling (1). Dex upregulates the transcriptional co-factor FHL2 in MSCs (1a) which interacts with β-catenin to increase its nuclear translocation (1b), resulting in increased RUNX2, ALP, and COL1 expression (1c). Another route of action is through activation of transcriptional coactivator TAZ (2), which stimulate OB differentiation through co-activation of RUNX2-dependent gene transcription, and inhibiting adipogenic differentiation through repression of PPARγ-dependent gene transcription. TAZ expression increase in response to BMP-2 suggesting a role in BMP-signaling. Dex increase TAZ expression (2a) leading to increased expression of BMP-2 and ALP (2b-c). Modified from Hamidouche et al 2008 and Hong et al 2009.*

Dex also stimulates human MSC migration (Yun et al., 2011) and preserves the proliferative capacity, multipotency and MSC phenotype through passaging (Xiao et al., 2010). Continuous 100 nM Dex treatment increase expression of apoptosis markers in human MSCs (Oshina et al., 2007), but is also demonstrated to inhibit contact-induced apoptosis in high density cultures (Song et al., 2009a). Other effects on human MSCs include suppression of cytokine (IL6, IL11) production (Kim et al., 1999), downregulation of PTH related protein and upregulation of PTH/PTHrP receptor (Ahlström et al., 2009, Zhou et al., 2011).

TABLE 2.2A. *Effects of dexamethasone on primary cultures of human bone marrow MSCs*

	Donor (n)	Donor age	Sex	Dex nM	CFU-F	Prolifer-ation	ALP	Mineralizatio n	Other effects of Dex
Beresford *et al.,* 1994	3	43-54	F/M	10	↑	↓	↑		
Rickard *et al.,* 1996		17-85	F/M	10			↑	↑	PTH receptor ↑
Fromigue *et al.,* 1997	10	Mean 66	F/M	10	↑	↑	↑	–	Col1 ↑
D'Ippolito *et al.,* 1999	41	7-70	F/M	10	↑	↓	↑	↑	
Walsh *et al.,* 2000	28	32-75	F/M	10	–	↓	↑		
Walsh *et al.,* 2001	30	33-77	F/M	10 100	– –	– ↓	↑ ↑		No effect on expression of a range of receptor molecules
Anselem *et al.,* 2002	31	27-53	F/M	10	↑	–			
Ogston *et al.,* 2002	1*	45	M	100		↓			
Leskelä *et al.,* 2003	43	52-92	F/M	100			↑	↑	Col1 ↑
Schecroun *et al.,* 2003	33	8-63		10	–			↑	

TABLE 2.2B. ***Effect of dexamethasone on osteogenic differentiation and mineralization of culture expanded human MSCs***

	Donors (n)	Donor age	Sex	Dex nM	Prolifer-ation	OB differentiation	OCN	Minera-lization	Other effects of Dex
Beresford *et al.*, 1994	3	43-54	F/M	10	↓	ALP ↑, BSP ↑	n.d.		
Cheng *et al.*, 1994	5	20-88	F/M	100	↓	ALP ↑	↑	↑	Continuous Dex treatment better mineralization than withdrawal
Fromigue *et al.*, 1997	10	Mean 66	F/M	10		ALP ↑, Col I ↑	–	↑	
Jaiswal *et al.*, 1997		10-58		10 100	 ↑	ALP ↑ ALP ↑		 ↑	Dose-response 1-1000 nM Dex: 10 and 100 nM best; 100 nM optimal
Kim *et al.*, 1999				10	↓	ALP ↑, Col I ↓			Dose-response 1-100 nM Dex: 10 and 100 nM best; 10 nM optimal.
Ogston *et al.*, 2002	1*	45	M	100	↓	ALP ↑, Col I ↓	–		PTH receptor ↑
Leskelä *et al.*, 2003	43	52-92	F/M	100		ALP ↑, Col I ↓		↑	
Pei *et al.*, 2003	8	56-80	F	10	–	ALP ↑	↑		
Mendes *et al.*, 2004	14	39-86	F/M	10		ALP ↑, Col I –			
Jorgensen *et al.*, 2004	18	21-27	F/M	100	↑	ALP ↑, Col I ↓	–	↑	
Nuttelman *et al.*, 2006	commer cial					ALP ↑, Runx2 ↑			
Chang *et al.*, 2006	7	17-77	F/M	100	↓	ALP ↑		↑	
Jäger *et al.*, 2008	3	20-46	F/M	10		Runx2 ↑, Col I ↑		↑	Decreased number of dead cells with Dex
Ahlström *et al.*, 2009	commer cial			10		ALP↑, Runx2↑	↓		Continuous Dex treatment better mineralization than withdrawal, PTH receptor ↑, PTHrP ↓
Hoemann *et al.*, 2009				10 100		ALP ↑ ALP ↓		↑ ↑	ALP and mineralization higher with 10 nM. Spontaneous adipogenic differentiation with 100 nM
Mostafa *et al.*, 2011	3	15-48		10 100	↑ ↑	ALP ↑ ALP ↑		n.d. ↑	100 nM Dex superior to 10 nM
Cordonnier *et al.*, 2011	17	37-80	F/M	100	↑	ALP –	–	↑	OSX ↓. Higher osteogenic differentiation and mineralization with BMP-4 than Dex

*Immortalized cell line from one donor; F/M: female/male; Dex: dexamethasone; ALP: alkaline phosphatase; Col I: collagen type I; ↑: stimulation; ↓: inhibition; – no effect compared to control without Dex; n.d.: no detectable levels. Only studies comparing conditions with and without Dex included. Read-out includes both gene expression and protein levels. Differences in time points for read-out not considered

2.3.5 Relationship between *in vitro* assayed MSC properties and *in vivo* function

The identity and nature of native MSCs in skeletal tissue and repair remains poorly understood. From a tissue engineering perspective, correlation between *in vitro* osteogenic capacity and *in vivo* bone formation after transplantation is essential and investigated with varying results (Mendes et al., 2004, Song et al., 2009b). How well the *in vitro* assayed properties of MSCs reflect the bone forming capacity of endogenous MSCs of donors is not known.

In vitro assayed MSC properties have been linked to demographic parameters. Decreased properties with increasing donor age are well documented. In addition, MSCs from patients with OP, OA and osteonecrosis show altered *in vitro* capacities. Glowacki and co-workers have demonstrated that *in vitro* osteogenic differentiation of MSCs correlate with vitamin D and PTH status of the donors. The expression of vitamin D metabolic enzyme in MSCs correlated with serum levels of 25(OH)D, 1,25(OH)D and PTH of the donors (Zhou et al., 2010a). MSCs from donors with low vitamin D levels demonstrated increased *in vitro* osteogenic differentiation, while correlating positively with serum PTH levels and whole body BMD of the donors (Zhou et al., 2012).

Clinical administration of PTH increases number and redistribution of MSCs in bone marrow (Ohishi and Schipani, 2011). Suh and co-workers (2012) demonstrated that magnetic resonance imaging can be applied to predict marrow cellularity and number of MSCs. Significant inverse correlation was reported between phenotypic *in vitro* mechano-response of MSCs and body mass index (BMI) of the

donor (Friedl et al., 2009b). The osteogenic mechano-response of MSCs also correlated with serum leptin and estradiol levels of the donors. Molecular analyses have provided valuable mechanistic explanations to the observations, contributing to understanding of the role of MSCs in age-and disease-related skeletal dysfunction.

In vitro observation has to be interpreted with caution, since the artificial conditions in culture are far from the complex systems present *in vivo*. Long-term cultures are associated with continuous changes in the global gene expression profile (Wagner et al., 2008). Recent data demonstrate dramatic changes in human MSCs gene expression patterns already during the first culturing step and under early passages (p1-3)(Qian et al., 2012). Microarray analyses revealed over 2-fold differential expression of more than 2700 genes in cultured compared to freshly harvested MSCs of the same donors. Changes were mostly seen in genes associated with surface receptors, including adhesion receptors, growth factors, matrix proteins and wnt-signaling, indicating possible alterations in differentiation potential and other cellular processes with culturing (Qian et al., 2012).

2.4 BONE QUALITY AND DETERMINANTS OF SKELETAL HEALTH IN POSTMENOPAUSAL WOMEN UNDERGOING THA

Bone quality is a frequently used term to collectively describe the multifactorial properties of bone tissue. As of today, there is no single definition to what bone quality is, how it can be measured in the most reliable way, manipulated, or how it should be taken into account in orthopaedic patients (Boskey et al., 2011). Qualitative and quantitative changes in skeletal and non-skeletal pathways with aging contribute to compromising bone quality. Dysfunction of MSCs and osteoblastic cells is emerging as the main cause of age-related bone loss. The bone quality of patients undergoing cementless THA defines the baseline conditions for healing of the joint implant.

2.4.1 Bone quality

Definition of bone quality

The term bone quality usually refers to bone strength and ability to resist fractures as an expression of sum characteristics (geometric and material properties and their interactions) (Fyhrie, 2005, Chappard et al., 2011). Currently there is no general agreement whether the term should include bone mass or not (Boskey et al., 2011, Hernandez and Keaveny, 2006). Bone quantity (i.e. mass) and quality together determines bone strength. There are several views on how these characteristics, separately and together, contribute to bone quality. A widely used definition is *all characteristics of bone tissue not accounted for by bone mass that contribute to bone strength* (Bouxsein, 2003, Donnelly, 2011). As any composite material, the quality of bone tissue is determined by its structural, chemical and physical properties explaining the variability in definition of the term with clinical, basic scientific and engineering point of views (**Box 4**). As a living material bone is metabolically active, and cellular and physiological aspects are crucial for the quality. A clinical definition of bone quality is "the mechanical and physiologic integrity of bone that may be compromised by disease states, altered homeostasis, nutritional, environmental, or pharmacologic agents" (Kinnett, 2011).

Whether bone mass assessed as the areal BMD measured by bone densitometry (DXA) should be considered a contributor to bone quality is debated. Partly due to the limited fracture risk assessment based on DXA alone. Paradoxically, DXA measured BMD is the most widely used method to clinically assess bone quality, and is in combination with plain radiography often the only method available. In this thesis, the term bone quality refers to the sum biological properties of bone, including cellular, metabolic and material properties contributing to skeletal health, strength and healing, including DXA measured BMD.

BOX 4. Parameters contributing to bone quality
(Bouxsein 2003, Brandi 2009, Jepsen 2011)

1) **Macroscopic geometry and size of whole bone**
2) **Microscopic architecture of trabeculae and osteons**
3) **Bone material properties**:
   - *Bone matrix composition*
   - *Arrangement of collagen and mineral*
   - *Volumetric bone density*
   - *Amount of microdamage, microstructural discontinuities, microporosity, connectivity*
   - *Remodelling dynamics*

Determinants of bone quality and age-related skeletal changes

Bone quality is affected by extrinsic factors including life style, nutritional factors, disease states and medications affecting bone formation and turnover. In addition, intrinsic factors including genetic inheritability and aging mechanisms influence bone quality (**Figure 2.8**). Skeletal aging is the main contributor to decreased bone quality in the general population, with menopause-related hormonal mass (Ammann and Rizzoli, 2003). bone. Depending on the mechanisms, basic properties of bone and its architecture can be altered changes causing additional deterioration in female affecting bone quality with or without affecting bone.

At the macroarchitectural level, size and shape are critical determinants, and especially the external diameters and cortical thickness affect whole bone quality. Increased external diameter increase the resistance to bending and torsional loads, while resistance to tensile and compressive loads is proportional to the cortical thickness. To resist the biomechanical requirements, the appendicular skeleton adapt by increasing the external diameter and decreasing the cortical thickness through periosteal apposition and endosteal resorption, respectively (Morgan and Bouxsein, 2008). At the microarchitectural level, structural patterns of trabecular and cortical bone determine the quality. Mechanical properties of trabecular bone depend on volume fraction and extent of anisotropy (Morgan and Bouxsein, 2008). Changes in microarchitectural structures deteriorate the mechanical quality of bone. The significant impact of microarchitectural properties is demonstrated by the disproportion in age-related reduction of bone mass in relation to skeletal weakening (Gabet et Bab 2011).

At the bone material level, quality is determined by matrix composition, mineralization and rate of turnover. Impaired enzymatic cross-linking and increased non-enzymatic crosslinking of collagen have adverse effects on mineralization and promote microdamage formation, deteriorating the biological and mechanical quality (Saito and Marumo, 2010). Degree of mineralization determine bone stability.

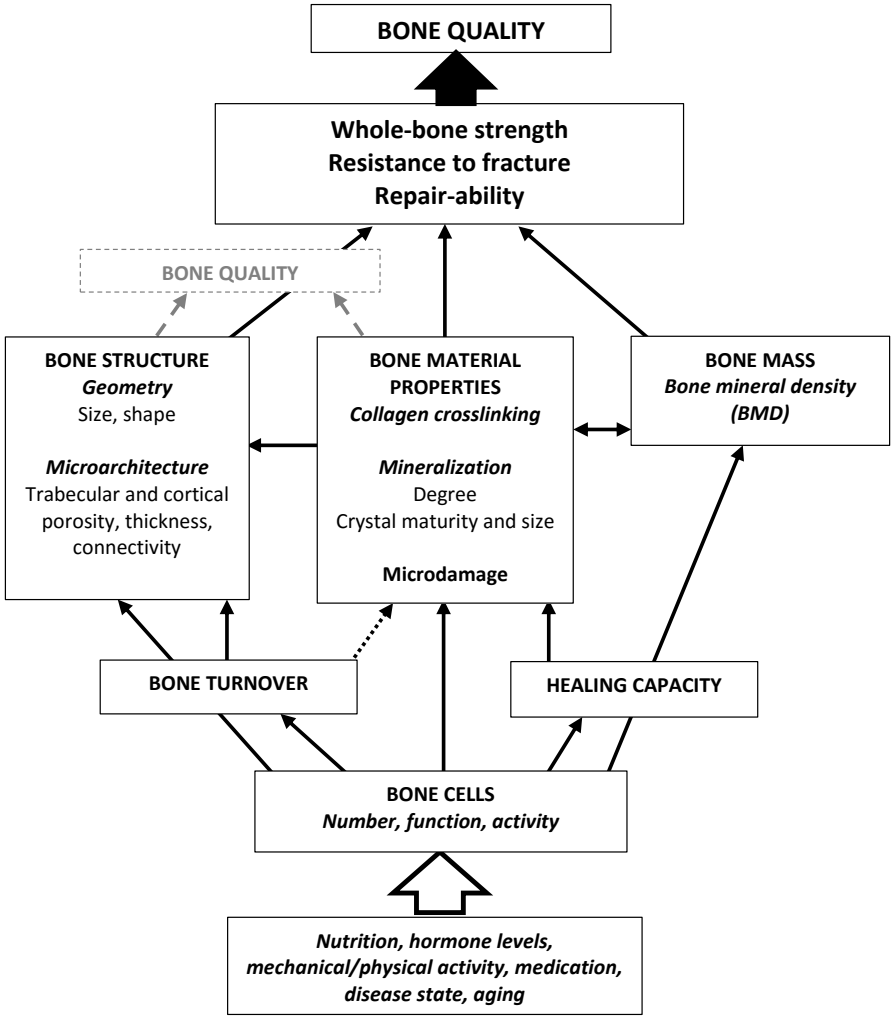

FIGURE 2.8 *Flow diagram showing factors constituting and contributing to bone quality. Adapted and modified from Jepsen KJ 2011 and Hernandez et Keaveny 2006.*

Enzymatic cross-link formation, degree of mineralization, and accumulation of microdamage are regulated by cellular activities. Availability and quality of MSCs and bone cells determine the rate of turnover and the quality of synthesized matrix. Imbalanced bone turnover results in decreased bone quality. High turnover reduces bone strength due to higher frequency of bone remodeling units altering the mechanical stress distribution. Low turnover alters bone geometry, degree of mineralization and inadequate repair, causing a buildup of microdamage (Seeman, 2008).

Since bone loss starts in both genders long before decline in sex-hormone levels, other age-related mechanisms contribute to aging of the skeleton (Demontiero et al., 2012). Many factors affect bone loss through mechanisms regulating MSC and OB number and function. Aging is associated with a general decrease in protein synthesis and turnover, causing accumulation of damaged proteins (Glowacki, 1999). Accordingly, levels of hormones and growth factors decline, with up to 14% per decade in elderly men and women (Rosen et al., 1994). Expression of pro-inflammatory cytokines increase with aging, negatively affecting bone turnover. Increased oxidative stress with aging is a fundamental mechanism causing decreased bone formation (Almeida, 2012).

VITAMIN D

Vitamin D (**Box 5**) and calcium are among the most essential nutritional factors affecting bone quality. The interplay between PTH, vitamin D, calcium and phosphate regulate serum ionized calcium levels within narrow limits required for normal neuromuscular function, bone mineralization and other essential physiological processes (Wacker and Holick, 2013). Vitamin D has multiple effects on bone, mainly as stimulator of formation, but also affects resorption (Parfitt et al., 1982, Wacker and Holick, 2013). Vitamin D further promotes bone health by maintaining physiologically healthy PTH levels.

BOX 5. Vitamin D synthesis and action

Inactive vitamin D (cholecalciferol) is obtained from conversion of 7-dehydrocholesterol in the skin by UV light, or by direct intake, and transported to the circulation bound to serum vitamin D-binding protein. In the liver vitamin D is converted into the pre-hormonal form 25-hydroxyvitamin D [25(OH)D] by hydroxylation followed by a second hydroxylation into active 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] by CYP27B1 hydroxylase (Holick et al., 1980).

The kidney is the richest source of CYP27B1 hydroxylase and responsible for the main production of active vitamin D for endocrine actions. CYP27B1 is also expressed by a number of extrarenal tissue cells including OBs (Howard et al., 1981), MSCs (Liu et al., 1999, Zhou et al., 2010, Geng et al., 2011), and immune cells (Adams et al., 1985, Weber et al., 2006) to provide 1,25$(OH)_2$D for intracrine or paracrine actions. Active vitamin D is taken up by cells expressing the intracellular vitamin D receptor (VDR), triggering a molecular cascade leading to activation of vitamin D target genes (Bikle et al., 2008).

Insufficient vitamin D levels can have deleterious effects due to reduced calcium absorption, decreased serum calcium and increased serum PTH, resulting in decreased BMD, muscle weakness, bone loss and increased fracture risk (Arabi et al., 2012). The non-skeletal actions include immune modulation (stimulation of innate and suppression of adaptive immunity) and involvement in insulin secretion (Bikle, 2008). Deficiency is associated with a range of disorders including autoimmune and infectious diseases. Considering the multiple skeletal and non-skeletal actions of vitamin D, deficiency might influence bone quality through several mechanisms. A recent study revealed new aspects how vitamin D deficiency reduce bone quality. Studying autopsy bone biopsies, Busse and co-workers (2013) found that vitamin D deficiency induce premature aging of bone in younger subjects.

There is no established consensus on what level of serum 25(OH)D constitute adequacy. Maintaining serum levels above 50 nmol/l (20 ng/ml) is the current therapeutic target recommended by the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (Rizzoli et al., 2014) and by the USA Institute of Medicine (Ross et al., 2011) in order to prevent deterioration of musculoskeletal health. Serum levels below 50 nmol/l are associated with increased bone turnover, bone loss and defects in mineralization (Rizzoli et al., 2014). However, serum levels ≥80 nmol/l (32 ng/ml) are needed for optimal calcium absorption, while <75 nmol/l (30 ng/ml) is the threshold for increased PTH levels and fracture risk (Holick, 2007, Heaney, 2011). The 75 nmol/l is a widely used deficiency cut-off and is the threshold recommended by the International Osteoporosis Foundation (Dawson-Hughes et al., 2010).

Serum vitamin D decreases with aging and deficiency is common in elderly (Lumachi et al., 2013). This can be due to decreased calcium absorption and vitamin D production by the skin, but also other mechanisms can be involved. Insufficient vitamin D contribute to impaired immunmodulation of the increased cytokine levels seen with ageing, indirectly contributing to decreased bone quality (Laird et al., 2010). Some studies have not found correlation between vitamin D and BMD (Glowacki et al., 2003, Lumachi et al., 2013), and the relationship seem to be dependent on age and PTH levels (Arabi et al., 2012). Vitamin D supplementation reduces bone loss and might prevent fractures, although appropriate supplementation is a topic of debate (Wacker and Holick, 2013).

PTH

As the master regulator of blood calcium levels, PTH significantly influences bone remodeling through complex actions. PTH synthesis and secretion from

the parathyroid glands is triggered by low levels of blood calcium and vitamin D, and high levels of blood phosphate. PTH promotes bone resorption to release bone calcium, induces renal preservation of calcium, secretion of phosphate, and stimulates renal production of $1,25(OH)_2D$ in order to increase intestinal absorption of calcium (Potts, 2005). Prolonged elevated level of PTH is associated with reduced bone mass and bone quality. The bone anabolic effects of intermittent low-dose PTH in healthy and osteoporotic subjects are therapeutically promising for improving bone quality (Esbrit and Alcaraz, 2013). In both men and women, serum PTH increases with aging. Both vitamin D and calcium deficiency can cause secondary hyperparathyroidism. A number of factors can cause age-related increase in PTH, including reduction in intestinal calcium absorption, impaired renal function and estrogen deficiency (Bullamore et al., 1970, Eastell et al., 1991, Demontiero et al., 2012).

ADDITIONAL AGING MECHANISMS CONTRIBUTING TO DECREASED BONE QUALITY

Increase in endogenous GCs affects bone through suppression and inhibition of OB activity (Weinstein, 2010). Increased serotonin level in older women is associated with decreased BMD (Modder et al., 2010). Decline in physical activity reduce mechanical loading and trigger a range of mechanisms contributing to further loss of bone mass and strength. COX-2 (cyclooxygenase-2) is the inducible regulator of prostaglandin E_2 and critical for normal bone repair. Age-related reduction in COX-2 can impair bone healing (Naik et al., 2009), and chronic inflammation may underlie healing problems with aging. Low levels of vitamin C causes impairment of collagen synthesis. Vitamin C deficiency is more common in elderly contributing to deterioration of bone microarchitecture and strength (Borrelli et al., 2012).

Accumulation of bone marrow fat at the expense of osteogenesis is a predominant feature of age-related bone loss (Meunier et al., 1971, Shen et al., 2007). The process starts during the third to fourth decade of life independent of hormonal changes (Perrien et al., 2006). The changed local conditions in the bone marrow stimulate adipogenic differentiation of MSCs (Abdallah et al., 2006, Zhou et al., 2008). Adipocyte secreted adipokines and fatty acids have toxic effects on OBs (Musacchio et al., 2007), and increased expression of PPARγ2 stimulates bone resorption (Lazarenko et al., 2007). Lipotoxic conditions can be one underlying cause of senile OP.

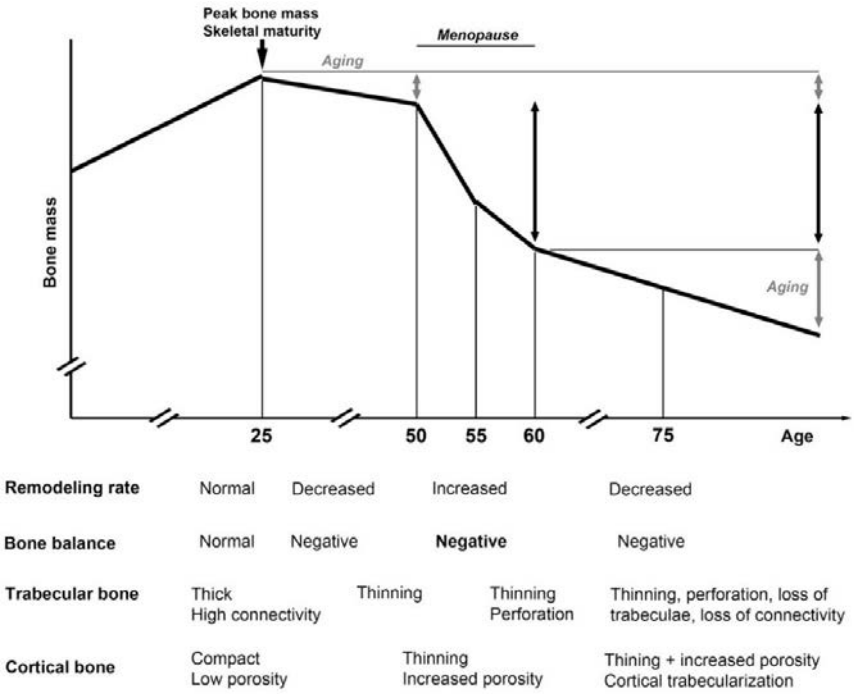

FIGURE 2.9 *Changes in bone mass and remodeling with aging and menopause along with structural changes in trabecular and cortical bone in women.*

2.4.2 Bone loss

*In women, two types of bone loss contribute to deterioration of bone quality with aging. A slow and continuous process of age-related bone loss is seen in both men and women starting when full skeletal maturation is reached (between 18 and 30 years of age), leading to an annual bone loss of approximately 1%. At menopause, estrogen deficiency triggers the more rapid postmenopausal bone loss, causing a 5-10% annual loss in bone mass (**Figure 2.9**) (Riggs and Melton, 1986, Marie and Kassem, 2011). During a lifetime, women loose approximately 50% of their bone mass.*

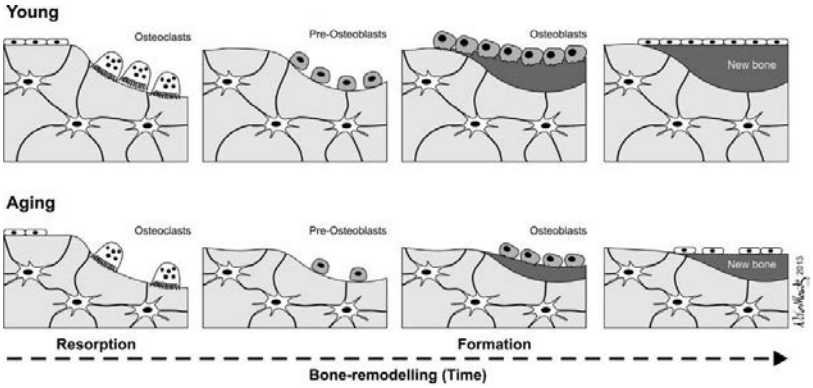

FIGURE 2.10 *Illustration of bone remodeling and its net effects in young and aging bone. Modified from Marie et Kassem 2011, Baron et Kneissel 2013. Illustration by Niko Moritz.*

Age-related bone loss

The slow phase of age-related bone starts through transition from tightly coupled and well balanced phase to a slower and negatively balanced one after peak bone mass and size is reached. The basic concept is decreased resorption volumes by OCs in each BMU, and slower birth rate of new BMUs, leading to decreased bone renewal and accumulation of microdamage (Lips et al., 1978, Seeman, 2008). In addition, reduced formation by OBs in each BMU accelerates the negative balance, resulting in net bone loss (**Figure 2.10**)(Marie and Kassem, 2011).

Postmenopausal bone loss

Upon menopause, estrogen deficiency cause additional bone loss through multiple mechanisms. Remodeling rate is increased through increased birth rate of new BMUs. Resorption volume increases and formation volume decreases as loss of estrogen prolongs the life span of OCs while reducing the life span of OBs (Eriksen et al., 1990, Manolagas, 2000) (**Figures 2.9 and 2.11**). The negative balance is further enhanced as bone is remodeled at all three endosteal envelops (endocortical, intracortical and trabecular)(Seeman, 2008). The high remodeling rate impairs the biological and mechanical properties. Interstitial bone too deep for remodeling become more densely mineralized and highly cross-linked with advanced glycation products.

Cortical and trabecular bone loss with aging

At the tissue level bone loss is seen as cortical thinning and increased cortical porosity, and as trabecular thinning, trabecular perforation and loss of trabecular connectivity (**Figure 2.9**). Consequences are unstructured and mechanically weaker bone (Zebaze et al., 2010). Dysregulation of remodeling does not occur with equal strength in all bone regions (Seeman, 2008).

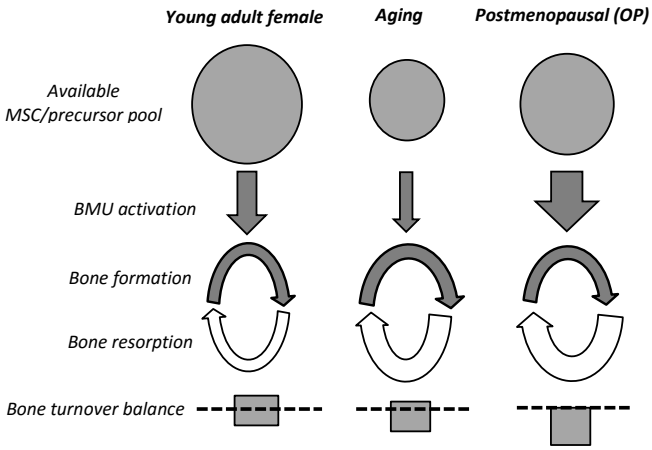

FIGURE 2.11 *Illustration of different effects of aging and menopause on bone turnover. Adapted and modified from Riggs and Parfitt 2005 and Sibai et al 2011.*

Due to the surface nature of remodeling, changes in bone mass occur earlier and to a greater extent in trabecular bone. Trabecular bone loss starts during the third decade, while the major loss of cortical bone takes place after the age of 50 and at higher rate in women (Ahlborg et al., 2003, Zebaze et al., 2010). In women, trabecular bone loss mainly takes place through trabecular perforation and eventually complete loss of trabeculae, leading to reduced trabecular number and loss of connectivity (**Figure 2.12**). In men, trabecular thinning predominates, maintaining the connectivity and number. Trabecular perforation and loss of connectivity reduce bone strength exponentially compared to equal volume lost through trabecular thinning (Aaron et al., 1987, Seeman, 2008).

Since only 20% of bone tissue is trabecular, most age-related bone loss is cortical. Remodeling at the endocortical and intracortical surfaces lead to increased porosity and trabecularization of the cortex (Zebaze et al., 2010) (**Figure 2.12**), significantly reducing the bone strength. The magnitude of age-related increase in cortical porosity has previous been underestimated, and is on possible factor affecting osseointegration of cementless THAs. Targeting cortical bone provide new approaches for preventing decrease in bone quality.

2.4.3 Age-related changes of proximal femur geometry

Changes in material properties leading to redistribution of cortical and trabecular bone contribute to the alteration of long bone geometry. In the proximal femur, age-related alterations in the three dimensional geometry is more drastic in females (Noble et al., 1995, Husmann et al., 1997, Casper et al., 2012). These changes are challenges when designing and choosing cementless femoral stems and may affect the outcome of the THA (Noble et al., 1988, Ahlborg et al., 2004).

Starting from the fourth decade, periosteal apposition slows down whereas endosteal resorption increases, resulting in increase of the outer diameter of long-bones and reduction of the cortical thickness (Heaney et al., 1997). During aging, this adaptive response compensates for bone loss in order to maintain whole-bone strength. The compensatory mechanism is stronger in men, making male long bone stronger compared to female. These gender differences with aging are mainly due to differences in bone size (Seeman, 2008, Jepsen et al., 2015). The increased remodeling rate in women at menopause causes further decline in cortical thickness, significantly affecting the mechanical properties of the entire femur (Zebaze et al., 2010, Ito et al., 2011). Whole bone strength can be therapeutically improved by increasing the external diameter through bone anabolic drugs which stimulates periosteal

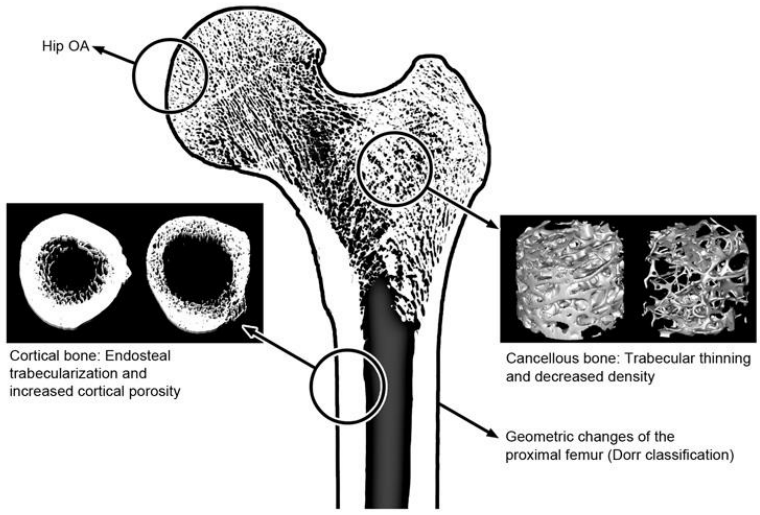

FIGURE 2.12 *Age-related changes in the proximal femur of female patients undergoing THA for hip OA. Several changes take place in the proximal femur potentially affecting the outcome of THA. Hip OA affect all tissue types of the joint driven by local inflammation. The quality of intertrochanteric cancellous bone and cortical bone in the femoral shaft decreases. Changes in turnover and material properties lead to alterations of femur geometry. Illustration by Niko Moritz.*

apposition, whereas cortical thickness can be increased with anti-resorptive agents, decreasing the endosteal resorption rate (Sibai et al., 2011).

Widening of the femoral canal diameter with age is well recognized from plain radiographs and autopsy studies (Smith and Walker, 1964, Noble et al., 1995). At younger ages, there is a significant difference in femoral canal diameter between men and women. After the age of 60, this difference disappears. Through endosteal resorption in aging women the canal width of female femurs approaches that of male femurs (Noble et al., 1995, Casper et al., 2012). The normally tapered shaped femur with thick cortices (type A) gradually change due to cortical bone loss. This leads to widening of the intramedullary canal diameter and thinning cortices (type B), with eventual complete loss of the tapered shape into a straight canal with thin cortices (type C). The annual increase in endosteal diameter in women is approximately 0.9% (Ahlborg et al., 2003). The female femoral shaft is more fragile since the increased inner diameter is not accompanied by increased outer diameter as seen in men (Zebaze et al 2010, Ito et al 2011). Changes in femur morphology is also under genetic influence, and have been linked to VDR genotype (Heaney et al., 1997).

In cementless THA changes to the femoral canal may adversely affect the fill and fit of femoral stems which have impact on early stability and increase the risk of perioperative and periprosthetic fractures. To ensure appropriate sizing and fit of femoral stems, several methods have been developed for evaluating the morphology from plain radiographs. Dorr classification (Dorr et al., 1993) and the canal flare index (CFI)(Noble et al., 1988) are widely used methods for approximating canal width prior to THA. Although computed tomography analyses provide more exact evaluation of the 3D femur morphology, the association between increased canal width and increasing age as determined from radiographs holds true (Husmann et al., 1997).

The Dorr classification is a simple system for qualitative evaluation of morphology of the proximal femur from conventional radiographs (Dorr et al., 1993). According to the classification system, there are three distinct femur types; A, B and C (**Figure 2.13**).

The canal flare index (CFI) was developed as a quantitative measurement of geometrical changes of the proximal femur (Noble et al., 1988). It is defined as the ratio of D to G, where D is the metaphyseal width 20 mm proximal to the most prominent point of the lesser trochanter and G is the width of intramedullary femoral isthmus (**Figure 2.14**). Index lesser than 3.0 corresponds to femurs with a straight

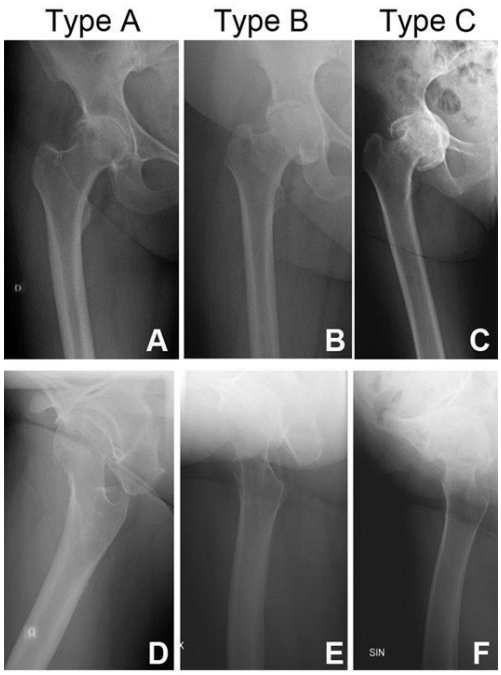

FIGURE 2.13 *Dorr classifications. Representative radiographs of Dorr type A, B and C proximal femurs in A/P (A-C) and lateral view (D-F).*

"stovepipe" shape (Dorr type C), index of 3.0 to 4.7 correspond to normal shapes (Dorr type A), whereas index over 4.7 corresponds to femurs with a marked proximal flare, designated "champagne-flute". The CFI was originally aimed as a guide for surgeons in the choice of femoral component for THA. CFI is only a measure of changes in canal width and do not take into account the thickness of the cortexes of the proximal femur.

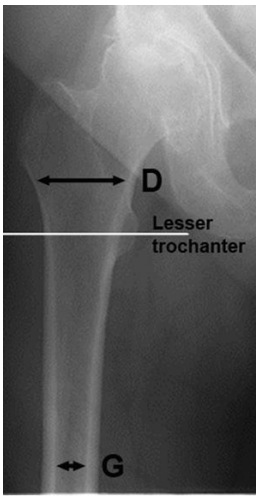

FIGURE 2.14 *Canal flare index is calculated from radiographs as the ratio of D to G, where D is the width 20 mm proximal to the most prominent point of the lesser trochanter and G is the width of the femoral isthmus.*

2.4.4 Osteoporosis (OP)

Osteoporosis is the most common bone disease in humans, traditionally defined as low bone density and microarchitectural deterioration of bone tissue making bones fragile and susceptible to fractures. Today OP is recognized as a far more complex disease, involving multiple mechanisms and pathways. Fractures are the major consequence of OP.

The current gold standard for diagnosing OP is based on the 1994 consensus statement from a WHO initiated expert panel. The classification is based mainly on DXA derived T-scores (**Box 6**), but also consider previous fracture incidence. **Primary OP** is loss of bone as part of aging, and is further classified based on underlying mechanisms. *Type I: postmenopausal OP* is accelerated bone loss due to decreasing estrogen levels in women after menopause. *Type II: senile OP* is referred to osteoporosis diagnosed in aged patients. **Secondary OP** is loss of bone caused by underlying conditions, diseases or medication, and **idiopathic OP** is defined in younger men and premenopausal women with osteoporotic fragility fractures without any secondary cause of OP (Armas and Recker, 2012).

BOX 6. Classification for diagnosing osteoporosis based on DXA measured T-scores according to current WHO criteria (1994)

Normal	T-score > -1.0
Osteopenia	T-score between -1.0 and -2.5
Osteoporosis	T-score between < -2.5
Severe osteoporosis	T-score < -3.5 or T-score < -2.5 with fragility fracture

The T-scores used as cut-off for identifying OP are derived from BMD and BMC measurements of 35-year old healthy population standards of respective gender as reference.

BMD of age-matched control populations have been used to calculate age-adjusted reference values (Z-scores).

PREVALENCE OF OP AND OSTEOPOROTIC FRACTURES

The prevalence of OP is continuously increasing due to the growing elderly population and modern life style. In 2010, patients with newly diagnosed OP in Europe was estimated to 22 million women and 5.5 million men, with a prospect of 135% increase by 2050. In Finland the number of osteoporotic patients in 2010 was estimated at 300 000 (~7%), of which women over 50 years of age are in majority. Worldwide, osteoporotic fractures was estimated to 9 million in the year of 2000, with the highest frequency in Europe (35%). The future estimates are contraindicative, but a stabilization rather than increase in fracture epidemic is expected (Svedbom et al., 2013).

PATHOPHYSIOLOGY AND RISK FACTORS

Osteoporosis is a heterogeneous disease with multiple pathogenic mechanisms. Irrespective of underlying mechanisms, four major processes contributing to decreased bone mass and increased fracture risk: 1) failure to achieve optimal peak bone mass and strength, 2) accelerated bone loss due to increased resorption, 3) decreased bone formation, and 4) increased fall incidence. All factors contributing to increased fracture risk are part of the pathogenic mechanisms. Usually a range of stronger and weaker factors contribute to development of OP. These include genetic predisposition, alterations in local and systemic hormones, life style and nutrition factors, and environmental factors (Raisz, 2005, Armas and Recker, 2012). Despite gender related differences in prevalence, bone loss and OP eventually affects all as part of the aging process.

MANAGEMENT OF OP

The outermost goal of OP treatment is preventing of fractures. Biologically, the aim is to increase the density and quality of bone, or at least to decrease or stop the bone loss. During the last ten years the treatment options for OP have increased, with several new medications of varying biological functions, targeting different parts of the bone remodeling complex. The main categories of pharmacological agents are antiresorptive aimed at inhibiting bone resorption, and anabolic aimed at stimulating bone formation (Rachner et al., 2011).

2.4.5 Hip osteoarthritis (OA)

OA is the predominant chronic joint disease in older adults, recognized as a slow progressive destruction of articular cartilage and subchondral bone accompanied by low-grade inflammation (Bijlsma et al., 2011, Loeser et al., 2012). The net effect is pain and deformity leading to joint failure. OA can affect any joint but is most common in knees, hips and hands. In the general population, hip OA is the most common cause of unbearable pain, decreased mobility and quality of life. Primary hip OA is the most common reason for THA in Western countries (Dagenais et al., 2009).

Osteoarthritis is recognized as a complex disease affecting all tissue types of the joint, including cartilage, bone, synovium, muscles and ligaments. Abnormal remodeling of joint tissues is driven by local inflammation as part of an active injury response rather than just a degenerative process (Loeser et al., 2012). OA is a multifactorial disease involving systemic patient-related factors (age,

gender, hormonal levels, genetics, and nutrition), intrinsic joint-related factors (variations in anatomy, muscle weakness, misalignment, laxity, and deformities), as well as extrinsic factors (rate and frequency of physical activities and obesity). Primary OA is defined as a condition developing in undamaged joints without a known cause. Secondary OA refers to conditions with well-defined causes, such as injuries, infections and anatomical abnormalities, and metabolic, developmental or neurological disorders (Bijlsma et al., 2011).

The major pathological changes in an OA affected joint include: 1) destruction and loss of articular cartilage, 2) subchondral bone alteration with sclerosis (deposition of new bone and callus formation) and cyst formation, 3) osteophyte formation at the joint margins (endochondral ossification at the bone-cartilage junction) and 4) synovial inflammation (Loeser et al., 2012). As the disease progresses the joint space is vanished, resulting in sever hip OA and need for THA. WHO has estimated that 80% of individuals with OA have limitation in movement, and 25% are unable to perform essential daily activities (Buckwalter and Martin, 2006). The primary symptom is pain, followed by stiffness and loss of movement and function.

DEFINITION AND DIAGNOSIS

Diagnosis is based on patient-reported symptoms, physical examination and radiological changes. There are no reliable methods for early recognition of OA. Laboratory tests have been developed with restricted success (Attur et al., 2013). There are several grading systems for radiographic definition. The most widely used is the Empire Rheumatism Council system presented over 50 years ago. This 5 scale grading system is often referred to as the Kellgren-Lawrence scoring. The American College of Rheumatology criteria for hip OA classification comprises several methods for diagnosis by different combinations of clinical, radiological and biochemical criteria (Altman et al., 1991). Several standardized questionnaires for definition and diagnosis of OA have been developed and designed specifically for individual joints. For hip OA alone there is a range of questionnaires and scoring systems, many available as computer-based to provide more efficient and reliable data collection (Shervin et al., 2011). The scoring systems are valuable for monitoring progression of hip OA and for evaluating surgical outcome. The most widely used disease scores are Harris Hip Score and WOMAC score (**Box 7**).

EPIDEMIOLOGY AND RISK FACTORS

OA is a public health concern worldwide, with corresponding sizable societal and economic burdens. In the western population >50% aged over 65, and 80% aged over 75 years are estimated to have radiologic OA. Symptomatic hip OA emerge at younger ages in men (from age 30-39 years), with increased prevalence in women after the age of 50. By ages 70-79 the incidences are similar. Age is the strongest risk factor, possibly reflecting decreased regenerative capacity and accumulation of risk factors. There are inconsistencies regarding the effect of BMI and obesity (BMI ≥30) on the risk for hip OA (Adatia et al., 2012, Jiang et al., 2012). Prior hip injury and vigorous physical activity are risk factors, in addition to certain hip anatomic abnormalities (Dagenais et al., 2009, Adatia et al., 2012).

BOX 7. Hip OA disease scores

Harris Hip Score (HHS) was developed to evaluate outcome of hip surgery (Harris, 1969). The outcome scores range from 0 to 100 points, evaluating pain (44 points), function (47 points), range of motion, deformity and limb length discrepancy (total 9 points). HHS was developed as a surgeon-assessed questionnaire, but has been validated for self-reporting. Higher scores are associated with less pain and better function.

The Western Ontario and McMaster Universities Arthritis Index (WOMAC) was developed in 1982 for evaluation of knee and hip OA (Bellamy et al., 1988). It is a self-administered validated outcome measure of 24 questions. It measures five items for pain (0–20 points), two for stiffness (0–8 points), and 17 for functional limitation (0–68 points). Lower scores are associated with less pain and stiffness, and better function.

2.4.6 Co-existence of osteoporosis and osteoarthritis

The original concept was an inverse relationship between OA and OP, where presence of OA was thought to protect against OP (Foss and Byers, 1972, Dequeker et al., 2003). Despite growing evidences of coexistence, the relationship between these two disorders remains debated.

Several reports demonstrate that prevalence of OP in the OA population corresponds to that in the general population (Healey et al., 1985, Karvonen et al., 1998, Glowacki et al., 2003). Hip OA has been suggested to reduce the fracture risk (Weintroub et al., 1982, Franklin et al., 2010), but also this is controversial (Arden et al., 1996, Calderazzi et al., 2014). The skeletal situation exposed during total hip arthroplasty usually reveals large differences in bone quality among postmenopausal women. It is not uncommon that these female patients have fragile cancellous bone both in the proximal femur and the acetabulum, suggesting that hip OA does not protect against OP.

Already in 1985, Healey et al. compared women with primary hip OA and age-matched women with primary OP and concluded that OP does not protect against hip OA. Dorr and co-workers (1990) found

that hip OA patients had lower cancellous iliac bone mass compared with controls. There is an increasing number of reports on the coexistence of the two conditions in postmenopausal women. Glowacki and her team (2003) found that 25% of postmenopausal women with hip OA undergoing THA had at least one T-score lower than -2.5 at any skeletal site measured. Later studies have confirmed that 20 to 29% of men and women with advanced hip or knee OA have OP and approximately 40% have osteopenia (Labuda et al., 2008, Breijawi et al., 2009, Lingard et al., 2010). A twin study confirmed an inverse relationship between OA and OP in the OA-affected hip, but found no such relationship between hip OA and BMD at other skeletal sites (contralateral hip, lumbar spine, total body) (Antoniades et al., 2000).

The inverse relationship is based on cross-sectional studies, often investigating BMD at the OA-affected site. Several early reports have suggested an increase in local BMD associated with OA compared to non-OA subjects (Burger et al., 1996, Arden et al., 1996, Cooper et al., 1991, Nevitt et al., 1995, Foss and Byers, 1972, Shen et al., 2009b), while others have found no differences in BMD between OA patients and healthy subjects (Hordon et al., 1993, Madsen et al., 1997, Sandini et al., 2005, Ding et al., 2010). When comparing femoral neck or trochanter BMD in control subjects (n=2012) and patients with hip or knee OA (n=99), Sandini and co-workers (2005) found no differences. In a DXA study of 19 female and 15 male patients with advanced unilateral hip OA, femoral neck and trochanter BMD of the OA affected hip was increased compared to the contralateral hip, whereas no difference was found in total hip BMD (Glowacki et al., 2010).

Geometry and composition of the bone are altered in OA affected hip or knee joints (Li and Aspden, 1997, Shen et al., 2009b), interfering with DXA measurements depending on the types of joint changes. BMD increase in presence of osteophytes, but not in case of isolated joint space narrowing (Antoniades et al., 2000). It is suggested that BMD of an OA-affected hip should not be used for diagnosing OP or predicting fracture risks (Setty et al., 2011). An early report suggested that OA is associated with increased trabecular BMD but not cortical BMD (Carlsson et al., 1979). The inverse relationship between hip OA and femoral neck fractures appears as one reason to the falsely suggested inverse relationship between the two diseases. Although "protected" against femoral neck fractures, patients with hip OA are susceptible to trochanteric fractures (Middleton and Ferris, 1996, Calderazzi et al., 2014). Higher total hip bone loss have been reported for patients with hip or knee OA compared to non-OA control subjects (Ding et al., 2010, Sandini et al., 2005). Taken together, these studies demonstrate a changed bone metabolism in OA patients, but also reflect the complexity of the disease which is not yet fully understood.

Mechanisms underlying the associations between OA and OP, as well as interactions of the two pathological conditions in a given individual are unknown. There is a range of common risk factors, including age, gender, physical activity, and vitamin D status. Other shared determinants include genetic, hormonal, and inflammatory factors. Both diseases have large genetic components (>50%), and genes for collagen type I, vitamin D receptor, estrogen receptor and wnt signaling all contributes to the pathogenesis of both OP and OA (Loughlin, 2001, Lerner and Ohlsson, 2015). Estrogens (Raisz, 2005) and leptin (Cirmanova et al., 2008) have impact on both cartilage and bone formation and homeostasis. Further, there are common inflammatory factors (IL-6, high-sensitivity C-reactive protein) associated with both OA progression and bone loss.

2.4.7 Age-related dysfunctions of osteoblast-lineage cells

Aging effects on MSC properties have been extensively studied and increasing evidences support age-related dysfunction in MSCs and osteoblastic cells as a main cause of bone loss in both genders after the age of 50 (Quarto et al., 1995, Zhou et al., 2008, Kassem and Marie, 2011). The relationship between aging of MSCs and age-related OP and OA is not known, but gene expression and miRNA analyses indicate disease-related changes in MSCs (Jiang et al., 2011). Age-related dysfunctions of OB-lineage cells can negatively affect osseointegration of cementless hip implants.

Age-related changes in fundamental osteogenic regulatory mechanisms contribute to cellular dysfunction. Alterations include decrease in number, proliferative and osteogenic differentiation capacity of MSCs, function and life-span of OBs, and increased cell senescence and apoptosis (**Figure 2.15**). Intrinsic alterations in human MSCs with aging contribute to skeletal aging. In addition extrinsic clinical factors cause age-related MSC dysfunction, and defective bone formation by inhibiting anabolic signaling pathways in bone (Zhou et al., 2008, Bellantuono et al., 2009). Wnt signaling regulate MSC self-renewal, proliferation, migration, and differentiation. Down-regulation of the Wnt/β-catenin signaling system is associated with senescence (Ye et al., 2007) and decreased differentiation (Brunt et al., 2012). With aging, there seem to be alterations in Wnt protein expressions (Shen et al., 2009a) and nuclear bioavailability of β-catenin in human MSCs (Brunt et al., 2012). Age-related dysfunctions in MSCs and osteoblastic cells are summarized in **Box 8**.

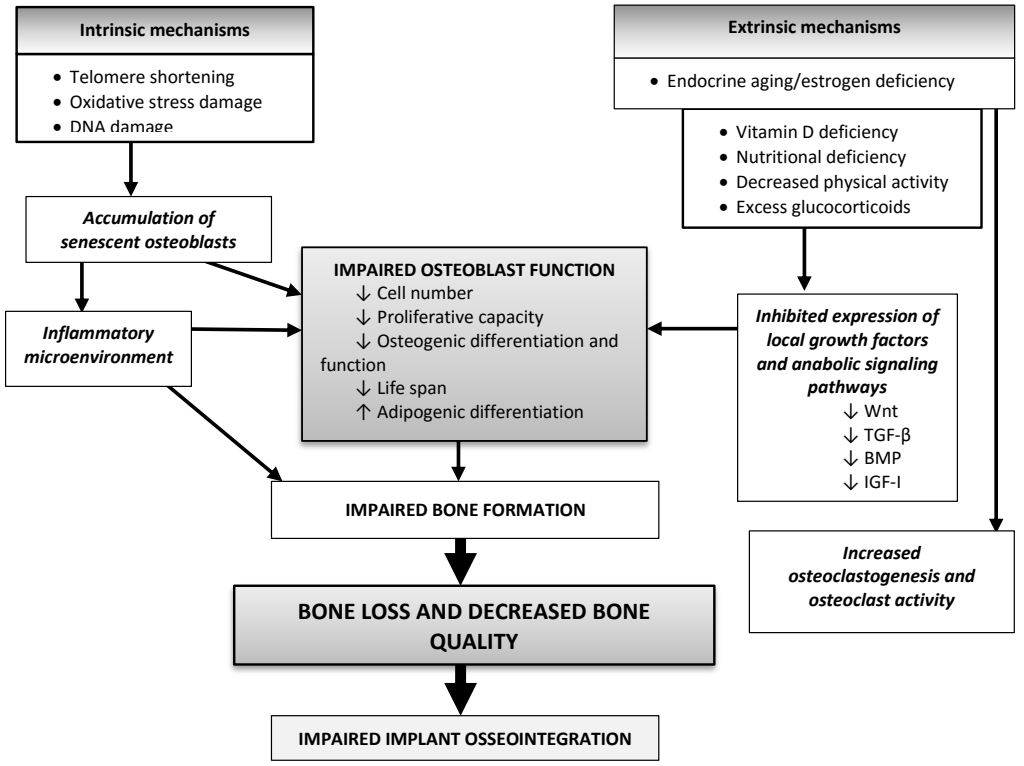

FIGURE 2.15 *Flow diagram of intrinsic and extrinsic mechanisms involved in age-related osteoblast-lineage cell dysfunction leading to decreased bone formation and decreased bone quality, potentially contributing to impaired osseointegration of cementless hip implants. Adopted and modified from Kassem et Marie 2011 and Marie et Kassem 2011.*

MSC dysfunction in OA and OP

MSCs from patients with OA and OP have gained much research attention in order to identify possible MSC-related disease mechanisms, and to evaluate the therapeutic potential of autologous MSCs in these diseases. Differences between MSCs derived from patients with OA or OP in comparison to healthy donors have been documented, but the role of MSCs in etiology and progress of the diseases is not clear. Interestingly, Jiang and co-workers (2011) found age-related up-regulation of genes associated with OA and OP in MSCs of aged healthy donors, indicating a pathological role of MSCs in these age-related skeletal diseases.

MSCs AND OSTEOARTHRITIS

The quality of MSCs from OA donors is unclear and there are conflicting reports. Murphy and co-workers (2002) found decreased chondrogenic and adipogenic differentiation of MSCs from hip and knee OA patients. Others have not found any differences in number, proliferation, or chondrogenic differentiation of OA MSCs. The osteogenic differentiation of MSCs from OA patients seem unaltered (Murphy et al., 2002, Scharstuhl et al., 2007, Jones et al., 2010).

Due to unknown causes, OA is associated with increased levels of TGF-β (Hopwood et al., 2007), which is also detected in MSCs (Rollin et al., 2008a) and OBs (Massicotte et al., 2002) from OA patients. Couchourel and co-workers (2009) found impaired mineralization by OA derived MSCs, attributed to abnormal collagen type I production secondarily to increased TGF-β expression. OA derived MSCs have an altered expression of VEGF superfamily members, possibly altering their angiogenic properties (Mwale et al., 2011), and an increased responsiveness to growth factors (Rollin et al., 2008b), indicating activation of OA MSCs in response to chemotactic signals, possibly due to licensing of the MSCs in the damaged tissue environment caused by OA. The number of MSCs in synovial fluid is increased in OA joints (Jones et al., 2008), correlating with the severity grading of OA (Sekiya et al., 2012). In addition, synovial fluid from OA joints promotes proliferation of MSCs from healthy donors when used as a culture supplement (Jones et al., 2008).

Immunological mechanisms are part of the disease and OA is associated with increased levels of inflammatory cytokines and matrix metalloproteinases causing cartilage destruction (Loeser et al., 2012). MSCs from OA patients have gene expression patterns enriched for antigen presenting and signaling pathways, down-regulation of several HLA class genes (Jiang et al., 2011), and increased IL-6 production (Hagmann et al., 2013), indicating possible involvement of MSCs in the immunological issues of OA.

MSCs AND OSTEOPOROSIS

MSCs from osteoporotic donors have been found to have decreased (Rodriguez et al., 1999) or unaltered proliferative capacity, and decreased osteogenic capacity (Zhang et al., 2009, Rodriguez et al., 2004), with some conflicting results (Stenderup et al., 2001). Differential activation of the ERK1,2 MAP kinase signaling pathway in MSCs from OP subjects can be one underlying mechanism (Rodriguez et al., 2004). In a comparison between MSCs derived from female OA and OP patients, growth kinetics were similar up to passage 5, but from passage 6 cell viability decreased significantly for OP MSCs (Zhang et al., 2009). The osteogenic capacity was higher for OA MSCs, while OP MSCs produced higher levels of OPG, possibly as a compensatory mechanism to the increased bone resorption environment in OP.

The role and involvement of MSCs in age-related skeletal diseases has been demonstrated in animal models, as reviewed in detail by Kassem and Marie (2011). Takada and co-workers (2006) demonstrated the therapeutic potential of MSCs in OP by using a well-characterized mouse model of accelerated aging with an OP phenotype (SAMP6). By intramedullary injection of allogenic normal bone marrow, the OP phenotype was treated. In contrast, an OP phenotype was created in normal mice by intramedullary injection of MSCs from SAMP6 mice (Ueda et al., 2007), that have been demonstrated to have dysfunctional MSCs (O'Sullivan et al., 2012).

BOX 8. Age-related changes in MSCs and osteoblastic cells contributing to bone loss and altered repair capacity (selected references)

Age-related decrease in MSC number	***Selected references***
The decline in MSC number suggested to correspond to different need for progenitors during growth and adulthood after completion of skeletal growth	Haynesworth et al., 1994
Six periods of differing MSC titers of decreasing repair capacity 1. Embryonal 2. Neonatal (Birth to 6 yrs) 3. Teens (~7-20 yrs) 4. Peak skeletal performance (20-30 yrs) 5. Midlife (~35-55 yrs) 6. Late life (~>60 yrs)	Caplan A 2004
The significance of reduced MSC number in age-related bone loss unclear, might be compensated for by MSC plasticity	Charbord P 2010
Decreased proliferation	
Significant age-related decline in proliferative capacity of MSCs well-documented for wider age range	Stenderup et al., 2003, Zhou et al., 2008, Alm et al., 2010, Brunt et al., 2012
Little additional decline in MSC proliferation after the age of 50-55 years. Corresponding to age periods of differing titers.	Schrstuhl et al., 2007, Leonardi et al., 2008, Fickert et al., 2010
Decreased proliferation of OBs with age. Caused by age-related alteration in membrane homeostasis and glycosylation of membrane components.	Reviewed in Kassem and Marie 2011
Altered expression of genes in glycosylation processes responsible for signal transduction and GF response	Jiang et al., 2011
Altered membrane glycerophospholipid composition and functionality	Kilpinen et al., 2013
Decreased proliferative capacity and increased senescence of MSCs in culture regulated by intrinsic aging mechanisms and not only a result of *in vitro* exhaustion	Zhou et al., 2008, Jiang et al., 2011
Increased senescence	
Increase in molecular pathways associated with senescence	Stenderup et al., 2003, Zhou et al., 2008, Jiang et al., 2011
Increased DNA damage	
Accumulation of cyclin-dependent kinase inhibitors	
Increased oxidative stress	
Progressive telomere shortening or modified telomere structures	Baxter et al., 2004, Jones et al., 2010
Decreased osteoblastic differentiation and function	
Osteogenic differentiation capacity of MSCs from elderly significantly lower compared to MSCs from younger donors	Zhou et al., 2008, Alm et al., 2010
Little additional decline in osteogenic capacity after the age of 50-60 years	Leskelä et al., 2003, Leonardi et al., 2008
Up-regulation of genes associated with inhibition of osteogenic differentiation in aged MSCs	Jiang et al., 2011
Down-regulation of genes associated with bone formation in aged MSCs	Jiang et al., 2011
Decreased life-span/Increased apoptosis	
Intrinsic and extrinsic mechanisms contribute to increased apoptosis with aging	Reviewed in Marie and Kassem 2011
Increased oxidative stress cause OB apoptosis	Reviewed in Almeida M 2012
Oxidative stress is accelerated by estrogen deficiency	Reviewed in Almeida M 2012
The antiapoptotic effect of vitamin D is lost in case of vitamin D deficiency	Geng et al., 2011
Age-related increase in pro-inflammatory cytokines induces OB apoptosis	Reviewed in Marie and Kassem 2011
Increased adipogenic differentiation	
Increased adipogenic differentiation at the expense of osteogenic differentiation of MSCs	Abdallah et al., 2006, Zhou et al., 2008
Age-related increase in the adipogenic transcription factors PPARγ2 and concomitant decrease in Runx2. A switch involving the action of leptin	Lazarenko et al., 2007 Thomas et al., 1999

2.5 ASSESSMENT OF BONE QUALITY

There is no single method for complete characterization of bone quality. Measurement of BMD is useful in diagnosing OP and patients with decreasing bone density, but provides no information regarding material composition, structural features or bone turnover. A combination of methods is required to obtain information on mechanical, microarchitectural and metabolic properties of bone. There are several effective and sensitive methods available for quantitative assessment of macro- and microstructure of bone, allowing evaluation of local and systemic skeletal health. Laboratory tests have been developed in order to provide additional and non-invasive assessment of skeletal pathology, especially in OP diagnostics and management. These methods are also useful for screening of postmenopausal patients scheduled for cementless THA.

2.5.1 Bone densitometry with dual-energy x-ray absorptiometry (DXA)

Bone loss and changes in BMD are not visible on plain radiographs. Dual-energy x-ray absorptiometry (DXA) is the established standard technique and the most widely used for measuring bone density. Additional techniques for bone density scanning include peripheral x-ray or ultrasound devices and quantitative computed tomography (QCT). The DXA technique, introduced in late 1980s, is the gold standard for diagnosing OP and estimating fracture risks in adults (Blake and Fogelman, 2010). The technique allows for measurement of bone density in the central and peripheral skeleton with good precision. Radiation dose is low (corresponding to natural background radiation), scanning is fast and the patient set up is easy. However, DXA also has essential limitations (Bolotin, 2007).

TECHNICAL PRINCIPLES

The DXA method measures the transmission of x-ray beams through the body. Attenuation of x-ray beams as they pass through a material is dependent on the initial photon energy of the beam, the mass attenuation coefficient (u_m) of the material and the mass per unit area of the material. In DXA, the use of two x-ray beams of different photon energies generates a dual energy. The difference in photon attenuation between the two energies at a given area is utilized to separate mineralized bone tissue from overlaying soft tissue (Pietrobelli et al., 1996, Blake and Fogelman, 2010). Energies are selected to optimize the separation. X-ray attenuation values are converted to bone mineral content (BMC, g) and bone area (cm) is calculated from the number of pixels within the region of interest. DXA reported BMD (g/cm^2) represents the *areal BMD* (BMD_a) and not the true volumetric bone density (g/cm^3). T-scores are used as cut-off for identifying OP (**Box 6**.)

PRECISION, ACCURACY AND LIMITATIONS

Precision of DXA measurements are usually expressed as coefficient of variations (CV) based on repeated measurements. For total hip and lumbar spine precision lies within 1-2 CV%, while CV for femoral neck, trochanter and ultra-distal forearm are around 2.5%. The accuracy of DXA, i.e. how close the measured BMD is to the ash weight of the actual calcium content, varies between 10-15%. Compared to biomechanically measured bone strength the two-dimensional areal BMD obtained with DXA corresponds to approximately 80% of bone strength (Blake and Fogelman, 2010).

DXA have certain limitations. Since DXA generates a 2D image of a 3D object the depth of bones is not accounted for. Therefore the technique is insensitive to size differences between subjects, causing variations with gender, ethnicity, size and weight. Sources of accuracy errors and artifacts are soft tissue, degenerative disease (especially in the spine) and marrow fat (Bolotin, 2007, Blake and Fogelman, 2010). Therefore, scan images need to be examined before interpreting the numerical results. Another problem is discrepancies between instruments from different manufacturers (Carey et al., 2007).

Decreased BMD with aging and disease is well correlated with increased fracture risk, but BMD alone is insufficient to predict fracture. BMD measurements detect impairment in bone mineralization but nothing regarding two of the most significant determinants of bone quality: material composition and structural design (Seeman, 2008). In terms of bone quality estimates, the two major limitations with DXA measured areal BMD are inabilities to 1) discriminate between cortical and trabecular bone, and 2) assess microstructure of bone. This is an issue since the rate of changes in response to aging, disease or medication is different in cortical and trabecular bone. Therefore measurement of one skeletal site cannot predict BMD at another site. DXA screening for OP might be unreliable in presence of hip OA causing high BMD values hence overestimating the bone density (Sandini et al., 2005, Glowacki et al., 2010). For identifying younger individuals at risk of sustaining fractures, DXA have lower value since similar BMD in young and old does not carry the same fracture risk (Licata, 2013). In a study on Swedish postmenopausal women, Ribom and co-workers (2008) found a two-fold increase in the number of patients diagnosed with OP when using a national T-score reference population.

CLINICAL ROLE

DXA measurements of BMD have three important clinical roles; OP diagnostics, evaluation of patients at risk of developing OP, and in monitoring the effect of anti-fracture treatment. Measurement of BMD at lumbar spine and hip are preferred, since BMD of the hip is the most reliable in predicting hip fractures, while spine BMD reflects response to antiresorptive treatment. For diagnosing OP in postmenopausal women and older men, combined hip and spine T-scores should be used according to IOF (Kanis and Gluer, 2000) and the International Society for Clinical Densitometry (ISCD). The ISCD recommends using the lowest T-score obtained from femoral neck, total hip and lumbar spine (www.iscd.org).

Since BMD alone is insufficient for proper fracture risk assessment, the WHO scientific group developed the Fracture Risk Assessment Tool (FRAX) (www.shef.ac.uk/FRAX) as an alternative and more accurate way for assessing the fracture risk. The FRAX tool combines clinical risk factors either alone or in combination with BMD, in order to prevent fractures and identify potential OP patients. The choice of BMD input to the FRAX model is one of the limitations (Licata, 2013, Setty et al., 2011).

2.5.2 Bone microarchitecture and mechanical properties

Measurements of bone structural characteristics is clinically difficult and knowledge on age-related changes in qualitative properties is largely based on autopsy retrievals or bone biopsies of limited size.

MICRO-CT ASSESSMENT OF BONE MICROARCHITECTURE

Traditionally, microstructural analyses are based on invasive bone biopsy techniques followed by histomorphometry *ex vivo*. Tedious histomorphometric methods of limited sample size are now often replaced by micro-computed tomography (micro-CT) analyses or corresponding high resolution computed techniques for nondestructive and rapid analyses of bulk tissue samples. Micro-CT has become the standard tool to quantify cancellous bone morphology and microstructure (Feldkamp et al., 1989, Burghardt et al., 2011, Boerckel et al., 2014), providing 3D measurements with realistic images of the microarchitecture and is nondestructive for the sample.

Analyses are performed in three critical steps including: I) scanning of bone specimen from multiple angles, II) reconstruction of the set of cross-sectional images into a 3D data set, and III) analyses of the data image set to obtain quantitative results. The system is composed of a sealed microfocus x-ray tube and a camera. For micro-CT scanning, small samples are placed in the sealed sample holder. A series of projection images is obtained by a rotation angle between each image. From the projection images a stack of 2D sections is obtained and reconstructed into 3D objects (Burghardt et al., 2011, Boerckel et al., 2014). Resolutions can be less than 10 µm/voxel.

Morphometric analyses of trabecular and cortical bone microarchitecture with micro-CT uses parameters derived from traditional histomorphometry (trabecular number, thickness, and separation) in addition to 3D parameters developed for micro-CT. Early evaluation studies comparing histomorphometry and 3D micro-CT has indicated strong correlations (Muller et al., 1998), while more recent reports indicate discrepancies (Chappard et al., 2007, Hordon et al., 2006). 3D measurements available for micro-CT data include degree of anisotropy (DA) which is a measure of the degree of structural orientation of the trabecular network, i.e., the extent to which trabeculae are aligned in a single direction (anisotropic), or randomly aligned (isotropic) (Odgaard et al., 1997). DA is highly related to the direction of mechanical loading. Skeletal sites subjected to a single loading direction have highly oriented trabeculae, hence high DA. Therefore DA varies between skeletal sites. Another 3D parameter is the structure model index (SMI) by which the shape of trabecular structures (rods and plates) can be characterized. Perfect plates, rods and spheres have SMI values of 0, 3 and 4, respectively (Hildebrand and Ruegsegger, 1997).

Numerous micro-CT studies on cadaveric bone specimens have demonstrated differing microarchitecture with aging, gender and anatomic locations (Burghardt et al., 2011, Boerckel et al., 2014). In clinical studies iliac crest or femoral neck bone biopsies are obtained for analyzing changes in trabecular bone structure as result of drug interventions, or metabolic and hormonal effects (Chappard et al., 2007, Hordon et al., 2006, Djuric et al., 2013).

One limitation of micro-CT is the requirement of small sample size. In older scanners the maximum sample size is approximately 36 mm in length and 14 mm in diameter, while newer scanners have approximate limitations of 140 mm length and 100 mm diameter (Donnelly, 2011). Micro-CT does not provide information regarding bone remodeling or cellular activity. For these parameters histomorphometry is required. Specimen shape strongly influence measurement outcomes and can provide misleading conclusions. The invasive methods reliant on biopsy specimens are not amendable for application in larger population based studies or as clinical routine. Several *in vivo*

applicable imaging techniques have been developed and evaluated for clinical imaging of bone microarchitecture. These include quantitative CT (QCT), high-resolution peripheral QCT, multidetector CT, high-resolution MRI and nuclear MRI (Donnelly, 2011, Burghardt et al., 2011). Recently, a novel pulse-echo ultrasound device (Bindex®, Bone Index Finland Ltd, Kuopio, Finland) has been developed, offering an attractive alternative for osteoporosis screening in primary health care (Karjalainen et al., 2016). The method is aimed at estimating proximal-femur BMD based on multi-site measurement of apparent cortical bone thickness and calculation of a normalized index based on the patient's age, body weight and height. This handheld device, which just received FDA approval, might be an efficient and inexpensive pre-screening tool.

MECHANICAL TESTING OF BONE

Mechanical testing of bone allows for characterization of multiple structural and material properties over multiple length scales (Donnelly, 2011). At the macroscale, whole bone mechanical testing can be performed using autopsy retrieved bones, and structural properties including stiffness and strength are assessed by loading a whole bone to failure in compression, bending or torsion. Outcomes are presented as *structural stiffness* representing the bone's resistance to elastic and reversible deformation, as *failure load* characterizing the strength of the bone, and as *energy absorbed to failure* representing a measure of structural toughness (Turner and Burr, 1993, Cristofolini et al., 2010).

Bone material testing at smaller length scale allows for assessment of intrinsic bone tissue properties including elastic modulus and ultimate stress force. Cortical and cancellous bone biopsies are typically used for characterization of mechanical properties of bone from different anatomical locations, or of different porosity, areal density, and mineral content (Donnelly, 2011). Mechanical testing of bone biopsies is also used as a read-out for evaluating the efficiency of therapeutic interventions aimed at improving bone quality.

For mechanical testing, regularly shaped specimens are prepared from cortical or cancellous bone biopsies. Specimens are typically prepared as cylinders or cubes with diameter or edge lengths of 5-10 mm. Specimens are tested to failure in compression, tension, bending or torsion, providing measures of effective elastic modulus and ultimate stress (Turner and Burr, 1993, van der Meulen et al., 2001). Results provide information on effects of porosity and geometric anisotropy on the mechanical properties of the bone tissue independent of the macroscopic characteristics and geometry of the whole bone.

2.5.3 Laboratory tests for assessing bone turnover and health

Laboratory tests provide non-invasive assessment of bone turnover processes and status. In addition to standard laboratory measurements several laboratory tests have been developed for more specific assessment of bone turnover. Bone turnover markers (BTMs) are divided into markers of bone formation and markers of bone resorption.

STANDARD LABORATORY MEASUREMENTS AS INDICATORS OF BONE HEALTH

Blood levels of PTH are measured as part of assessment of bone metabolic status. Measured together with calcium and vitamin D levels, primary, secondary, or tertiary hyperparathyroidism can be distinguished. Due to the effects of PTH on both kidney function and bone, serum calcium, phosphorus, and creatinine levels are usually measured together with PTH. Since the pro-hormone 25(OH)D is released back into the circulation, measurements of its serum levels is considered the most reliable indicator of vitamin D status (Hewison et al., 2007).

BONE FORMATION MARKERS

Collagen type I. OB-synthesized procollagen molecules are processed extracellularly to form mature collagen. As part of the process the C- and N-terminal ends of the procollagen molecules are enzymatically cleaved, producing C-terminal propeptides (PICP) and N-terminal propeptides (PINP). These peptides are released into the circulation and can be measured as biomarkers using immunoassays. Since collagen type I is abundant also in a range of other tissues, PICP and PINP are not bone specific. Due to short half-lives in the circulation (at the order of minutes) the within day variation can be high (Cremers et al., 2008).

Alkaline phosphatase *(ALP).* The ALP enzyme is cleaved off the OB membrane and released into the circulation, allowing measurement of ALP activity from serum samples. The bone isoform of ALP represents approximately 50% of serum levels, whereas the other half is liver derived (Green et al., 1971). Since the clearance from blood is slow, the half-life of ALP in serum is several days making the within day variation low compared to other BTMs. Commercial kits available for measuring bone ALP use monoclonal antibodies for specific detection of the bone isoform, although there is a 20% cross reactivity with the liver isoform (Seibel, 2005).

Osteocalcin derived molecules can be measured as a reflection of bone formation. Intact OCN is rapidly degraded in serum, but different fragments have

longer half-lives (Garnero et al., 1994). There are numerous assays are available for measuring intact OCN or different fragments (Cremers et al., 2008).

BONE RESORPTION MARKERS

Pyridinium cross-links. In mature collagen, the structure is stabilized by amino acid cross-links between the triple helical fibrils. In collagen type I and other fibrillar collagens there are mainly pyridinoline and deoxypyridinoline cross-links, which are released into the circulation as degradation products from bone resorption. Free or peptide-bound cross-links can be measured in serum and urine using immunoassays or HPLC (Naylor and Eastell, 2012).

***Cross-linked telopeptides of type I collagen*.** Other collagen degradation products from bone resorption are peptide fragments that also are released into the circulation. The telopeptides of collagen type I are non-helical fragments containing the cross-linking regions (Cremers et al., 2008). Both the N-terminal cross-linked telopeptide (NTX-I) and the C- terminal cross-linked telopeptide (CTX-I) can be measured in serum and urine using immunoassays.

***Tartrate resistant acid phosphatase (TRACP)*.** TRACP isoenzyme 5 is expressed by macrophages and OCs. Activated macrophages express the 5a isoform, while the 5b isoform is predominantly expressed by OCs (Janckila et al., 2002). TRACP5b is an intracellular enzyme with two enzyme activities; an acid phosphatase activity and a reactive oxygen species generating activity. TRACP5b functions are not well understood, but degradation of internalized bone matrix components through one or several mechanisms have been suggested (Halleen et al., 1999, Angel et al., 2000). TRACP5b serum levels increase during growth and in pathological conditions associated with increased bone resorption (OP, hyperparathyroidism) (Capeller et al., 2003). Serum levels of TRACP5b can be measured with immunoassays and reflects the OC number (Halleen et al., 2000).

LABORATORY TESTS AND SERUM MARKERS OF BONE TURNOVER AS DIAGNOSTIC TOOLS

Although BTM measurements are widely assessable today, for measurements to become standard laboratory tests there are still a range of challenges to solve. Due to the many sources of variation, reliable interpretation of the results is difficult. Patient characteristics (basic demography, diseases and medications), sampling parameters (time of the day, sample handling and storage) and assay-related parameters are all sources of variability. BTMs also correlate with 25(OH)D and PTH levels. Low vitamin D and high PTH are associated with increased BTM levels, reflecting stimulation of bone turnover. Lack of established reference ranges is another limitation. There are several BTM tests available with varying specificity and sensitivity. Serum marker levels seem less variable than urinary levels (Naylor and Eastell, 2012).

BTM measurements are especially useful in detecting secondary causes of OP, by identifying patients with high turnover, and can predict major osteoporotic fractures (Bonjour et al., 2014). The value of BTMs in predicting bone loss is unclear. At the cohort level, BTMs are inversely associated with BMD and rate of bone loss (Lenora et al., 2007), but have little predictive value at the individual patient level (Naylor and Eastell, 2012). The correlation between BTMs and BMD is stronger in postmenopausal women compared to younger women (Rosen et al., 1997). So far, BTMs seem useful for monitoring progress of OP and osteopenia, and for evaluating response to therapy. In a position statement the International Osteoporosis Foundation recommends serum PINP for formation and serum CTX-I for resorption as reference markers in research and clinical studies for comparable results across studies (Vasikaran et al., 2011).

2.6 CEMENTLESS HIP IMPLANT BIOLOGY

2.6.1 Osseointegration

Biological fixation of cementless hip implants occurs through a series of biological events resembling fracture healing (Galante et al., 1971) and is referred to as osseointegration (Branemark et al., 1977, Albrektsson et al., 1981). Successful osseointegration of a hip implant is characterized by cessation of micromotion (Mann et al., 2012).

The originally histological term osseointegration was defined as "*the direct structural and functional connection between ordered living bone and the surface of a load –carrying implant*" (Branemark et al., 1977, Albrektsson et al., 1981), based on the finding by Brånemark and co-workers (1969), describing the formation of a direct interface between titanium implant and bone, without intervening soft tissue. A more clinical definition is: "*a process whereby clinically asymptomatic rigid fixation of alloplastic materials is achieved, and maintained, in bone during functional loading*" (Zarb and Albrektsson, 1991).

There are two major phases in the life time of a cementless THA; the first weeks and months when the implant need to be firmly fixed (primary fixation/stability), and the rest of the life time when the implant need to stay fixed (secondary fixation)(Bauer and Schils, 1999). Primary fixation relies on mechanical interlock achieved at time of surgery, while secondary fixation relies on a firm biological fixation achieved through bone apposition and ingrowth to the implant surface. To function normally, the implant needs to obtain a permanent stable fixation to bone, which relies on the quality of the secondary fixation. This in turn depends on a rigid primary fixation to allow for proper bone ingrowth. Early disruption of the osseointegration process leads to loss of primary stability and early migration, whereas late failure of the process result in implant loosening.

Biology of the osseointegration process

Despite the high annual number of orthopaedic implants utilized clinically for decades, the process of osseointegration of long-bone implants remain unclear. While osseointegration of dental implants have been extensively studied and described in detail (Davies, 1998), knowledge on the biological fixation of orthopaedic implants largely relies on studies of fracture healing (Gerstenfeld et al., 2003) and autopsy retrievals (Sychterz et al., 2002, Engh et al., 1995). Biological details are mainly derived from experimental animal studies and *in vitro* experiments.

Biological fixation of a cementless hip implant occurs in response to a surgical trauma, triggering an acute inflammatory reaction in bone and surrounding soft tissues, activating the osteogenic processes (Jacobs et al., 2000). The principle mechanisms are similar to the cascade events in fracture repair, although the tissue response is modified by the implant characteristics, stability of the primary fixation, and intraoperative injuries. The major events in the osseointegration cascade include: hematoma formation, mesenchymal tissue development, transient woven bone formation, lamellar bone formation, reestablishment of bone marrow, and continuous remodeling of the interface (Goodman et al., 2009).

Upon implantation primary stability is attained through mechanical press-fit, but a varying degree of gaps is generated between the implant surface and the surrounding bone due to anatomical variations of the proximal femur, stem design and surgical technique. Bone ingrowth into these spaces are required to achieve secondary stability. The early osteogenesis around an implant proceeds in two directions: from the host bone towards the implant – *distance osteogenesis*, and from the implant toward the healing bone – *contact osteogenesis* (Davies, 1998).

The early stage of osseointegration involves protein adsorption, platelet activation, coagulation and inflammation. Immediately after positioning of the hip implant, proteins from blood and tissue fluids adhere to the surface facilitating attachment for cells, and possible changes to the implant surface chemistry (depending on material and coating). The biological events are regulated by growth and differentiation factors released by activated blood cells. Inflammatory cells (polymorphonucleated leukocytes, monocytes and macrophages) are recruited and activated to secrete factors (e.g. IL-1, IL-6, TNFα) affecting inflammation and bone healing. This also stimulate recruitment and activation of OCs that resorb dead bone fragments and cellular debris, which can further compromise the primary stability (Goodman et al., 2009).

Platelets activated in response to the foreign material aggregate to the surface. The interaction of blood cells with implant induces clot formation during the first days after surgery and coagulated blood fills the void volume around the implant, forming a fibrin matrix providing an osteoconductive scaffold for MSCs and OB precursors. The surgical bone injury stimulates recruitment of MSCs and preOBs from the marrow and endosteal surface, colonizing the implant from day one after implantation (Marco et al., 2005). MSCs and preOBs adhere to the fibrin matrix and secrete cytokines, growth and differentiation factors including FGFs, TGFβ, BMPs, IGFs and VEGF (Albrektsson and Hansson, 1986, Davies, 1998), stimulating

proliferation, differentiation and maturation of OB precursors (Jacobs et al., 2000, Goodman et al., 2009). The MSCs and early OBs deposit a thin (0.2-0.5 μm) non-collagenous matrix rich in calcium, phosphate, osteopontin and bone sialoprotein, promoting OB attachment. This afibrillar layer initiate appositional *de novo* bone formation characterizing contact osteogenesis.

As the acute inflammation phase resolve (within a few days), OBs start to produce osteoid onto the implant followed by rapid intramembranous woven bone and trabecular callus formation, creating a first anchoring of the implant and offering resistance to early loading (Goodman et al., 2009). The wide and active surface area is connected with marrow, providing necessary vasculature, MSCs and OC precursors. Contact osteogenesis is 30% faster than distance osteogenesis, and contribute more to the biological implant fixation. In a successful osseointegration processes, remodeling starts the subsequent weeks, substituting woven bone with stronger, highly mineralized, and more load resistant lamellar bone (Jacobs et al., 2000). Initial osseointegration takes approximately 4-12 weeks, but progressive bone ingrowth can continue over several months, and even up to 3 years (Galante et al., 1971, Zweymuller et al., 1988, Khanuja et al., 2011). Remodeling at the bone-implant interface continues throughout life.

Although providing useful basic mechanisms describing the biological process of osseointegration, data from dental implant healing is not entirely applicable to long-bone implants. The fundamental differences between jaw- and long-bone healing and remodeling are commonly underestimated when applying knowledge from dental implant healing to orthopaedic implants. The obvious discrepancy in biomechanical conditions is only part of the distinction. As previously reviewed by Matsuura and colleagues (2014), the jaw along with other craniofacial bones arise from neural crest cells of the neuroectoderm germ layer in contrast to the mesoderm derived skeletal stem cells of the appendicular skeleton. Neural crest derived cells represent a more immature cell source higher up in the "stem cell" hierarchy, as demonstrated by higher osteogenic potential and broader differentiation potency of MSCs from the jaw. Consequently, regulatory mechanisms and functional characteristics differ between long-bone and jaw-bone MSCs.

Factors affecting osseointegration

A range of biological, physical, chemical and thermal factors, together with regulators of the tissue response determines the outcome. The requirements for proper biological fixation are;

1) Intimate initial contact between implant surface and host bone
2) Minimal relative motion at the implant-bone interface
3) Appropriate surface characteristics of the implant
4) Osteogenic potential of the implantation site.

It is well established that lack of direct contact between implant and bone negatively affect bone ingrowth and strength of fixation, independently of stability of the implant (Kienapfel et al., 1999). Proper mechanical conditions (initial contact, minimal relative motion) are prerequisite for the biological processes to take place, whereas surface properties of the implant and quality of the host bone contribute to the quality of cellular responses and secondary fixation, ultimately determine the overall success or failure of the implant.

Osseointegration is enhanced by implant related factors (design and chemical composition, surface topography and coatings, extent of coating, material, shape, length, diameter), and alterations in implant properties are aimed at providing optimal mechanical stability and loading conditions as well as providing an implant surface that can accelerate the biological processes at the bone-implant interface leading to permanent fixation. Although implant parameters are crucial for a successful outcome, the physiological and cellular responses dictate the fixation process. Bone quality at the implantation site affect initial stability of a press-fitted cementless THA and can together with the bone forming capacity determine the long-term success. If appropriate stem cells are not present, osteogenesis is inhibited (Jacobs et al., 2000, Kassem and Marie, 2011). Hence, the quality of the surrounding bone bed and its healing capacity is ultimately responsible for a proper response to the implant (Engh et al., 1987, Mavrogenis et al., 2009, Goriainov et al., 2014). Despite bone ingrowth, failure of fixation can occur as a result of fatigue fractures of the bridging trabeculae (Jasty et al., 1991), further indicating the importance of good bone quality around the implant for long-term survival and function.

Factors inhibiting osseointegration include excessive micromotion at the interface, suboptimal porosity of implant coatings, pharmacological agents (e.g. COX-2 selective inhibitors non-steroid anti-inflammatory drugs, NSAIDs), and patient-related factors including OP, rheumatoid arthritis, increased age, and nutritional deficiency (Bobyn et al., 1987, Mavrogenis et al., 2009, Soballe et al., 1993b, Vuolteenaho et al., 2008). New approaches under investigation for enhancing osseointegration of THA includes bone marrow grafting, biocoating of implants with growth or differentiation factors (e.g. BMPs, PDGF, IGF) or extracellular matrix proteins (e.g. collagen, fibronectin, vitronectin), and systemic administration of pharmacological agents (e.g bisphosphonates and PTH) (Agarwal and Garcia, 2015).

PRIMARY STABILITY AND MICROMOTION

For osseointegration to take place, load transfer must take place with minimal micromotion at the bone-implant interface. Failure of implant fixation might be due to relative motion between implant and bone tissue. Firm primary stability and sufficient osseous contact of cementless stems with surrounding bone (i.e. mechanical interlock acquired at surgery) minimize micromotion favoring bone ingrowth (Galante, 1971). To achieve best possible initial fixation, a slightly over-sized femoral component is used and inserted by press-fitting.

Mechanically unstable conditions around cementless implants result in fibrous tissue formation and inhibited bony ingrowth. In case of fibrous fixation, continuous micromotion at the implant-bone interface can cause displacement inhibiting osseointegration, eventually leading to aseptic loosening and implant failure. By studying controlled implant motions in an experimental large animal model, Bragdon and co-workers (1996) demonstrated that micromotion less than 20 µm results in predominantly bony ingrowth, while micromotion of 40-150 µm leads to a mixture of fibrous tissue and bone formation. Motion at the interface exceeding 150 µm predominantly results in fibrous tissue formation and minimal osseointegration (Engh et al., 1992, Pilliar et al., 1986, Jasty et al., 1997). Studies of cadaveric bone have demonstrated micromotion up to 350 µm at the bone-implant interface of radiographically fixed cementless stems (Sychterz et al., 2002). Accordingly, mechanical instability of the stem may result from two different conditions: stem loosening (macro-instability) or fibrous fixation (micro-instability). Although mechanical instability is not always radiologically detectable, it is clinically associated with increased thigh pain (Engh et al., 1987). In worst cases the solution is revision.

Detailed autopsy studies of stable osseointegrated cementless hip implants showed that on average only 35% (±5%) of porous coated surfaces had bone ingrowth (Engh et al., 1995). At the biologically fixed areas, cortical bone grew circumferentially into the porous coatings, and was integrated with the outer bone cortex. A more recent autopsy study applying high-resolution digital image analysis reported bone-implant contact fractions ranging between 25% and 72% for radiologically stable stems (age 58-92 years). The degree of interface micromotion was inversely proportional to the amount of bone-implant contact (Mann et al., 2012).

Clinically, evaluation of osseointegration is based on interpreting radiographs without definitive proof of mechanical stability. Based on plain radiographs cementless stems can be classified as osseointegrated, fibrous stable or unstable based on qualitative radiographic features including bone bridging (spot welds), reactive lines, pedestal formation, calcar remodeling, radiolucent lines, implant position and migration exceeding 4 mm. Semi-quantitative radiological assessment can be done according to a fixation/stability scoring system (Engh et al., 1990). Stems that show spot weld are considered osseointegrated. This definition of osseointegration is merely based on empiric knowledge of clinical component stability, and histological examination of autopsy retrievals (Engh et al., 1992, Sychterz et al., 2002).

As a way of assessing implant stability and detecting motion, the position of the femoral stem can be measured manually from repeated radiographs using anatomical landmarks. This provides information of possible stem sinking and disalignment, indicating instability. The manual method is robust, and a change of position of approximately 5 mm is needed for identification (Sutherland et al., 1982). The EBRA method (Ein Bild Röntgen Analyse) provides a computerized method for calculating implant migration from plain radiographs. The method has an accuracy of 1-1.5 mm (Biedermann et al., 1999, White et al., 2012). Radiostereometric analysis (RSA) is the most accurate method for measuring implant migration. With an accuracy of 0.2 mm and ability to calculate three dimensional migrations RSA has revolutionized bone implant research (Selvik, 1974, Kärrholm et al., 1997, Nelissen et al., 2011).

OSSEOINTEGRATION IN AGING AND OSTEOPOROSIS

Numerous animal studies have shown slower osseointegration of both dental and orthopedic implants in experimental OP (Vandamme et al., 2011). *In vitro* studies using human or rodent OBs and MSCs have clearly demonstrated impaired proliferation, differentiation and bone formation of cells derived from aged and osteoporotic subjects, cultured either on plastic or different implant materials (Olivares-Navarrete et al., 2012, Giro et al., 2015). Age-related changes in bone biology can have impact on implant osseointegration and long-term mechanical stability. While osseointegration of dental implants have been widely studied, demonstrating impaired or slower implant healing in aging and OP (Omar et al., 2011, Giro et al., 2015), much less in known about osseointegration of orthopedic implants in OP patients and there is no definite data on survival of cementless THA in these patients.

Previous studies have investigated the effect of local bone quality (BMD of operated hip, Singh Index, hip fracture as sign of local OP) on clinical and radiological outcome of cementless THA. Kirsh *et al*. (2001) found no difference in clinical and radiological evaluation of a HA-coated THA between patients with OP bone (Singh Index 1-3) and non-OP bone (Singh Index 4-6). Patients were over 65 years of age and

follow-up was 2-10 years. Similar results were reported by Rhyu *et al.* (2012), comparing clinical and radiological outcome of a cementless double-tapered femoral stem in younger (< 50 years) control patients and older (>70 years) patients with T-score of proximal femur less than -2.5.

Fully coated cementless femoral stems have displayed minimal 2-year migration (< 1 mm) in aged (65-92 years) patients with femoral neck fractures (Skoldenberg et al., 2011b, Figved et al., 2012). Both these studies reported good clinical outcome at 2 years. A 5-year follow-up indicated good stability but high periprosthetic bone loss and late-occurring fractures (Skoldenberg et al., 2014). In a third study (Schewelov et al., 2012), major subsidence (average 3 mm) was documented during the initial 3 months in hip fracture patients aged 70-96 years old, but stems stabilized thereafter with good 2-year outcome.

2.6.2 Stem design

Stem design is decisive for biological and biomechanical integration of the implant with the bone. It affects the strain distribution of the proximal femur after THA and thereby the biological response. There is a large variety of cementless stem designs available on the market, differing in terms of geometry and mechanisms for obtaining initial fixation, but with similar successful survival rates (Khanuja et al., 2011). Design and surface patterns together designate the fixation of the stem.

Initial mechanical stability achieved at time of surgery is critical for long-term fixation. This is influenced by geometry and surface properties, while material, design and surface dictate the long-term stability (Khanuja et al., 2011, Carli and Jerabek, 2015). The goal is to develop stems to achieve more stable initial fixation for faster and more consistent bone ingrowth to secure the long-term outcome.

In cementless THA, stem geometry important for load transfer to the femoral bone stock, affecting bone remodeling. Pattern of stress distribution and bone reactions is determined by the stiffness between bone and implant, the extent of coating, and the alignment of the prosthesis (Bobyn et al., 1992, Engh et al., 1995, Decking et al., 2006). By using materials with a lower modulus of elasticity and by altering the geometric design, stiffness is reduced and thereby stress shielding can be reduced. Isoelastic stems with the same modulus of elasticity as human femoral bone were developed to decrease stress shielding (Butel and Robb, 1988, Nistor et al., 1991). However, the more flexible stems caused an increased proximal stress at the interface, deteriorating bone bonding, causing micromotions at the interface with subsequent implant loosening (Huiskes et al., 1992). Cementless stems are usually made of cobalt-chromium-molybdenum alloys (CoCrMb) or titanium-aluminum-vanadium alloys (Ti-6Al-4V)(Khanuja et al., 2011). Since the modulus of elasticity of titanium alloys is closer to that of bone most femoral stems today are made of Ti-6Al-4V (Carli and Jerabek, 2015).

The basic stem designs are straight or curved. These can be cylindrical, tapered or anatomic. The shape can further be wedged or modular (Khanuja et al., 2011). There are also short-stem designs. The two central properties by which cementless stems can be identified are 1) geometry through which initial stability is achieved (anatomic, tapered, or straight) and 2) where and how the stem is achieving permanent bone fixation (type and extent of roughness and coating (Carli and Jerabek, 2015). **Figure 2.16** demonstrate the development of cementless stem designs.

Extent of fixation and thereby amount of stress shielding is determined by the extent of porous coating. Fully coated stems more often show radiological signs of stress shielding compared to proximally coated stems. The more proximal the fixation, the lower the stress-shielding (Engh and Bobyn, 1988). To better achieve initial stability and dependable fixation in elderly women with altered femoral morphology and osteoporotic cortical degradation, modular (Sporer and Paprosky, 2004), custom-made (Mulier et al., 2011, Santori and Santori, 2010) and short-stem (Khanuja et al., 2014) femoral implants have been evaluated. Variable ranges of periprosthetic bone loss and stem migration have been reported for cementless stems. The length of the stem also affects the pattern of remodeling and osseointegration. With longer stems, proximal strain is reduced while distal strain is increased (Arno et al., 2012). Hence, with longer cementless stems, the load transfer is less physiologic and lead to increased proximal bone loss due to stress shielding. Although cementless stems of variable designs have been used and studied for a long time there are still no definitive conclusions about optimal designs for different situations (age, activity level, bone geometry type, deformities). Therefore, repoting stem designs is important for comparison of outcomes. With well-designed and well implanted cementless femoral stems stable fixation and osseointegration can be achieved also in aged patients. Stress-shielding and thigh pain are remaining concerns driving further development.

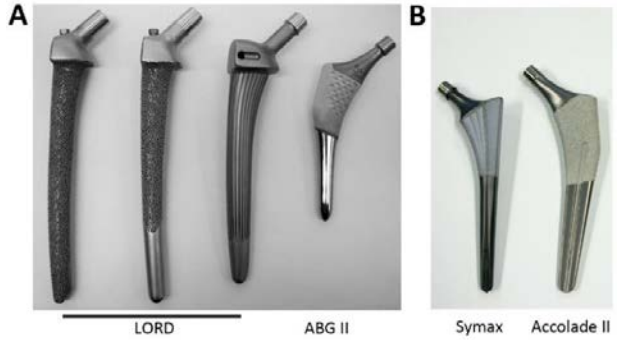

FIGURE 2.16 ***Evolution of cementless stem designs.*** *(A) LORD cementless stems represent early designs introduced in the 1970's. The first stem design had extensive porous coting (left), followed by modified versions with less (middle) and no (right) porous coating. The anatomic ABG II stem (Stryker) with proximal HA coating was introduced in 1996. (B) More recent stem designs introduced during the 21st century represented by the Symax stem (Stryker) with anatomic proximal geometry and straight design with proximal HA coating. The Accolade II stem (Stryker) has a tapered wedged design.*

2.6.3 Implant surface

The biological response to cementless implants is largely affected by the implant surface (Eldridge and Learmonth, 2000). Therefore, efforts for improving the bone-implant interface follow two approaches: physical improvement by altering the surface topography and chemical improvement by biochemical surface modifications (Dohan Ehrenfest et al., 2010). The stimulatory effect of porous and roughened surfaces on osseointegration is well established. The implant surface properties regulate the osteogenic differentiation of MSCs (Olivares-Navarrete et al., 2010).

SURFACE TOPOGRAPHY

With a porous surface, bone grows inside the surface structures, i.e. bone ingrowth. For bone ingrowth to occur, pore size needs to be 50-400 µm (Albrektsson et al., 1981, Haddad et al., 1987), with an optimal pore size between 100 and 400 µm (Bobyn et al., 1980a, Cook et al., 1987). In case of a roughened surface, bone grows onto the surface, i.e. bone ongrowth.

Cementless fixation benefits from rough and porous surfaces in several ways. The increased surface topography increase the contact area between implant and bone tissue (Haddad et al., 1987). Porous coatings and roughened surfaces also provide a high friction surface for best possible initial fixation (Albrektsson et al., 1981) contributing to desirable immediate mechanical interlock (Bobyn et al., 1980b).

Numerous *in vitro* studies have demonstrated variations in cell adherence, proliferation, differentiation and matrix production between different surface materials, topographies and coatings. Surface topography and roughness of the implant stimulates favorable cellular responses enhancing osseointegration. The roughened surface promote attachment of adhesion proteins from the surrounding tissue and body fluids, thereby providing attachment sites for MSCs and preOBs (Anselme et al., 2002, Borsari et al., 2005). The roughened surface itself also stimulates expression of appropriate integrins at the cell surface (Raz et al., 2004), further promoting cell attachment. The stimulatory effects of surface topography on osteogenic differentiation of human MSCs is through activation of wnt-signaling (Galli et al., 2012). Microrough surfaces activate the non-canonical wnt pathway (Olivares-Navarrete et al., 2011), while HA-coating is a strong activator of the canonical wnt pathway (Thorfve et al., 2014).

HYDROXYAPATITE COATING

To further enhance osseointegration HA coating of porous hip implants was introduced in the mid-1980s (Furlong and Osborn, 1991). The benefit is thought to be mediated as both improved quality and timing of bone ingrowth (Soballe et al., 1993a). Successful HA coating is dependent on chemistry and crystallinity creating a balance between bone stimulating ion release and long-term stability of the coating (Dumbleton and Manley, 2004). Experimental studies showed that HA-coating have positive effects on implant fixation in various situations (Soballe et al., 1999) and allow for a less perfect initial fit within the bone (Soballe et al., 1993a). Bone ingrowth have been demonstrated in gaps up to 1 mm (Soballe, 1993), even under loading conditions (Overgaard et al., 1998, Mouzin et al., 2001), and in OP and osteopenia, or revision (Soballe et al., 1993a, Hara et al., 1999, Soballe et al., 2003).

Although HA coatings have been in clinical use for more than 20 years, advantage in cementless stems is debated. Clinical studies comparing HA coated and non-coated stems of the same design have demonstrated no difference in revision rates or clinical outcome (Yee et al., 1999, Rasquinha et al., 2002, Kim et al., 2003, Yoon et al., 2007, Lazarinis et al., 2011). In measurements of stem migration using RSA (Soballe et al., 1993b, Luites et al., 2006) or EBRA (Ein Bild Röntgen Analyse) (Hamadouche et al., 2001), less subsidence have been demonstrated with HA-coated stems compared to uncoated stems of the same design, whereas analyses of plain radiographs have failed to show this differences (Incavo et al., 1998, Yee et al., 1999, Rasquinha et al., 2002, Kim et al., 2003).

2.6.4 Radiostereometric analysis (RSA) for monitoring osseointegration

Radiostereometric analysis is the most accurate technique available for measuring implant migration, allowing three-dimensional monitoring of micromigration (Selvik et al., 1983) and can detect hip implant movements down to the magnitude of 0.2 mm and rotational movements of 0.5 degrees, depending on the RSA set-up (Soballe et al., 1993b, Kärrholm et al., 1997, Bottner et al., 2005). In vitro studies have demonstrated accuracy of 0.021 mm to 0.12 mm (Onsten et al., 2001, Bragdon et al., 2002). RSA provide a useful method for monitoring migration and detecting stem loosening early postoperatively (Kärrholm et al., 2006, van der Voort et al., 2015). RSA indirectly measures stem fixation, as osseointegration is characterized by cessation of micromotion.

Radiostereometry

BACKGROUND AND BASIC PRINCIPLES

Plain radiographs provide limited information on early implant migration. Traditional radiological features of loose stems (subsidence, pedestal formation, cortical hypertrophy and increasing radiolucency) appear at a late stage. Migration has to exceed 5 mm for identification (Sutherland et al., 1982) and it can take up to 10 years before final signs of femoral stem loosening appear on conventional radiographs (Pijls et al., 2012). RSA was presented by Göran Selvik 40 years ago (1974) based on previous radio photogrammetry and mathematical principles (Davidson and Hedley, 1897) developed for evaluation of metallic implant fixation (Bjork, 1968, Hallert, 1954). Since then, the RSA method has been continuously developed to improve the performance and accuracy (Kärrholm et al., 1997, Valstar et al., 2005). The basic principle of RSA is to determine the precise position of two objects relative to each other in three dimensions. By determining the relative position of the femoral component and the proximal femur, implant migration can be detected from repeated examinations (Selvik, 1974). RSA investigation includes four main steps: 1) implantation of tantalum markers into the bone segment, 2) radiographic examination, 3) computer aided measurements from radiographs, and 4) calculations of movements (**Figure 2.17**).

RSA is mainly used in assessment of prosthetic fixation. Other emerging applications include monitoring fracture stability (Ragnarsson and Kärrholm, 1991, Madanat et al., 2012), implant wear particle migration (von Schewelov et al., 2004, Callary et al., 2013) and femoral head penetration (Bragdon et al., 2004). In addition RSA has been applied to study joint kinematics (Uvehammer et al., 2000), skeletal growth (Hagglund et al., 1986), and for evaluating transdermal femoral implant systems in above-the-knee amputations (Nebergall et al., 2012).

TECHNICAL PRINCIPLES

The method is based on the geometric concept of rigid bodies, which is a mathematical model described by a point matrix. According to mathematical kinematics, a rigid body is a system of mass points in which the distance between all paired points remain constant throughout motion (Euler, 1776). In the matrix, any three points that are non-collinear form a rigid body. If the distance between the three points remains constant, the exact position of the rigid body can be calculated. If the movement of a rigid body in space is such that every point on its matrix has the same movement, the movement is defined as translation. If all points on the rotation axis remain constant, and all other points move relative to their distances from this axis, the movement is defined as rotation (Bottner et al., 2005). In RSA, the movement of one rigid body (implant) in relation to another rigid body (bone) is plotted in an artificial reference coordinate system. This is achieved by obtaining simultaneous dual radiographs in combination with a calibration cage constituting a 3D reference coordinate system (**Figure 2.17A-B**). For calculating the exact position of each rigid body (implant and bone) within the matrix, the coordinates of three landmark points is needed for each rigid body segment. Tantalum beads are inserted into the implant and surrounding bone to be used as distinct landmarks.

TANTALUM MARKERS FOR SEGMENT LOCALIZATION

Tantalum metal is an ideal material for the purpose of RSA. Its high biocompatibility and resistance to corrosion makes it optimal for implantation into the human body, whereas its high atomic number makes it easy to identify on radiographs. The tantalum beads for RSA are implanted permanently into the bone, and have not been associated with any adverse reactions (Kärrholm et al., 1997). For implantation into the body segment, tantalum beads with diameters of 0.6 mm and 0.8 mm are used. For adult hip joints, 0.8 mm beads are commonly used, whereas the 1.0 mm beads are used for marking the more radio-dense implant. Beads are inserted to the bone using a spring-piston (Aronson et al., 1974). For rigid body calculations of the bone segment, a minimum of three non-collinear markers are required (Selvik, 1974, Valstar et al., 2005). To guarantee successful analysis, insertion of 6 - 9 markers into the bone segment is recommended to compensate for loose or invisible markers. The markers need to be randomly distributed over the bone segment, with clear proximal-to-distal separation to enable their identification (**Figure 2.17C-D**). Accuracy of RSA increases if markers are placed in a manner that creates a larger rigid body (Ryd et al., 2000). Optimal approximate positions of bone markers in a clinical study can be predetermined through *in vitro* studies using

phantom models (Mäkinen et al., 2004, Bragdon et al., 2004, Madanat et al., 2005)

The implant segment is marked with tantalum beads during manufacturing. Due to manufacturing issues, the number of implant markers is kept at a minimum and usually only 3 markers are incorporated. For RSA of femoral stem implants, the center of the femoral head is readily used as an addition marker (Valstar et al., 2005). The calibration cages used for reconstruction of the reference coordinate system contains a number of 1.0 mm tantalum beads located in fixed and well-defined positions in the walls of the cage.

RADIOGRAPHIC EXAMINATION

Radiographic examinations are performed by simultaneous exposure to dual x-rays. For this set-up, several radiographic arrangements can be used. The most commonly used are uniplanar and biplanar techniques. In biplanar set-up the two x-ray recording media (films, cassettes, digital) are placed at a 90-degree angle to each other, whereas in the uniplanar set-up, the x-ray recording media are placed side-by-side (Valstar et al., 2005). The two x-ray tubes can be ceiling mounted or mobile. In the uniplanar set-up, the x-ray tubes are angled approximately 20˚ in relation to the floor and 40˚ in relation to each other (Kärrholm et al., 1997)(**Figure 2.17A**). For THA the uniplanar set up is used. Radiation doses are equivalent to or lower than corresponding conventional radiography. The calibration cage (**Figure 2.17B**) is placed on top of the roentgen films, under the patient examination table (**Figure 2.17A**). The calibration cage contains tantalum markers at well-defined positions in order to create a three-dimensional coordinate system in which displacement of the patient markers are calculated.

ANALYSIS OF RADIOGRAPHS

3D position of bone and implant are computed from digital radiographs using computer software (**Figure 2.17C**). Several software packages have been developed. The calibration cage provides the global coordinate system. When calculating relative motion, a reference rigid body has to be selected, which is based on the clinical question. In prosthetic motion calculations the host bone is the reference segment (Valstar et al., 2005).

Accuracy of subsequent calculations of micromotions is affected by the distribution of the tantalum beads within the rigid body, and their stability across follow-up (Kärrholm, 1989). For proper calculations, tantalum markers need to be non-collinear, randomly and largely distributed over the segment. This is ensured for each examination by calculating the **condition number (CN)**, which is an indicator of marker distribution and a measure of how well markers are scattered. High CN indicate marker positions close to linear, representing poor distribution, whereas a low CN reflects wide spatial

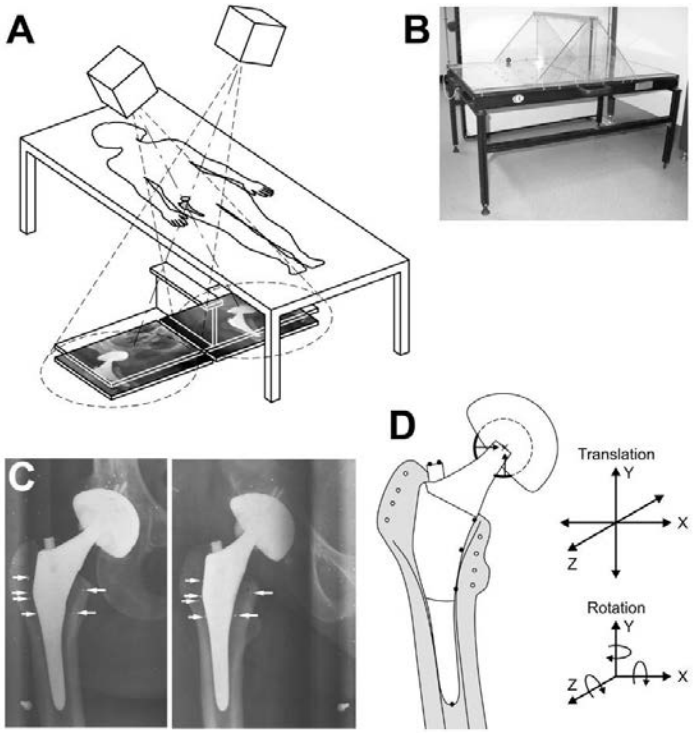

FIGURE 2.17 RSA imaging and measurements of micromotions. (A) Schematic drawing of radiographic examination of the operated hip with simultaneous exposure of two X-ray units. UmRSA calibration cage (B) with X-ray plates is placed under the examination table. (C) Postoperative radiographs of a prosthesis with visible RSA bone markers in the surrounding proximal femur indicated with arrows. (D) Schematic drawing of the prosthesis with RSA tantalum markers on the implant and in the surrounding bone. The center of the head can be used as an additional marker. The right-hand coordinate system for analysis of three-dimensional stem migration in relation to the femur is indicated

distribution. Condition numbers below 110 are considered very reliable and 150 is the upper CN limit according to RSA guidelines (Valstar et al., 2005). Stability of tantalum markers between follow-up examinations can be assessed by calculating the **mean error of rigid body fitting (ME)**, representing the mean difference between the relative distances of markers in a rigid body in one examination compared to another examination. The upper limit for ME is 0.35 mm according to RSA guidelines. ME and CN calculations constitute important quality control parameters that have to be confirmed before proceeding with calculation of micromotions from follow-up examinations.

CALCULATION OF MICROMOTIONS

Translations of implants are presented as motions in the distal-proximal (Y), medial-lateral (X), and posterior-anterior (Z) directions (**Figure 2.17D**). Set in the right-hand side of the body, translations are converted into anatomical relevance by designated positive X as medial, positive Y as superior and positive Z as anterior. Left hand extremities are dealt with by reversing X-axis motions and presented in terms of anatomical direction. In femoral stem migration, negative translation along the Y-axis is usually referred to as stem subsidence, or stem sinking to better explain the clinical situation (Kärrholm et al., 1994). Accordingly, rotation about the Y-axis corresponds to external-internal, or anteversion-retroversion rotation. Rotation about the X-axis corresponds to flexion-extension (X), and rotation about the Z-axis is abduction-adduction. Positive rotations are set for right hand extremities, while rotations in left hand extremities are corrected by converting Y- and Z-axis directions (Valstar et al., 2005).

As an expression of total translational and rotational migration, vectors can be calculated using the three dimensional Pythagorean Theorem ($T^2 = X^2 + Y^2 + Z^2$) (Selvik, 1974, Kaptein et al., 2007). Migration vectors can only have positive values. A commonly used parameter is the maximum total point motion (MTPM), which represents the length of the translation vector of the point in a rigid body that has the greatest motion. MTPM is supposed to reflect the biological process contributing to (implant) motion. However, MTPM not only depends on the amount of motion, but on the location of the point and can differ between implant designs, between follow-up examinations, and is sensitive to marker instability (Nilsson and Kärrholm, 1993, Valstar et al., 2005). Hence, MTPM is a less reliable parameter compared to segment motion parameters and is not good for comparisons between studies and implant designs.

ACCURACY AND PRECISION

Accuracy of RSA is defined as the closeness of agreement between micromotion measured by RSA and micromotion measured by a method of higher resolution (Ranstam et al., 2000, Ryd et al., 2000). Accuracy measurements are performed using *in vitro* phantom models, and are required when setting up new RSA facilities or introducing new RSA methods. Several phantom studies have demonstrated high accuracy of RSA for THA (Onsten et al., 2001, Bragdon et al., 2002, von Schewelov et al., 2004, Bragdon et al., 2004, Bojan et al., 2015).

Precision is defined as the closeness of agreement between repeated measurements and is assessed by double examinations within a time-interval of 10 to 15 min, with repositioning of the patient between the examinations. In clinical studies, precision is evaluated for each patient (Ranstam et al., 2000, Valstar et al., 2005). Precision values are commonly used as detection limits to define whether the segment of interest (implant, fracture) is fixed or migrating. Due to high accuracy and precision of RSA, clinical trials can be performed with small patient populations (Ryd et al., 1995, Valstar et al., 2005).

CHALLENGES, LIMITATIONS AND FUTURE PERSPECTIVES OF RSA

Although the basic principles are relatively straightforward, the practical application of RSA is more complex. Radiographic examinations and calculation of micromotions are time consuming. The method is technically demanding, accounting for several sources of measurement errors (Derbyshire et al., 2009), and use of different RSA set-up systems account for additional variability. Results can be presented and interpreted in a number of ways (mean, median, range, maximum migration) and differences in detection limits and methods for determining thresholds further contribute to variations between reports. As a first step towards standardization, a European RSA group published user guidelines in 2005, including a list of standardized output for clinical RSA studies (Valstar et al., 2005). However, a recent systematic review demonstrated that although guidelines have improved the methodological reporting of hip and knee arthroplasty RSA studies, adherence to guidelines are still low (Madanat et al., 2014).

To overcome manufacturing challenges associated with attachment of tantalum markers to implants, model based RSA techniques have been developed. By matching the radiographic projection of the prosthesis to a virtual projection of a three dimensional model, implant position can be assessed without tantalum markers (Valstar et al., 2001, Kaptein et al., 2003). The accuracy of model based RSA is considered comparable to traditional RSA (Hurschler et al., 2009). We recently demonstrated that although the accuracy of model based RSA was not as high as for marker-based RSA, the clinical precision is acceptable (Farsani et al., 2016). The technique provides increased possibilities for RSA

evaluation of new implant designs. However, the model based technique requires that the implant is asymmetric in order to obtain a unique three dimensional projection, which is a challenge with some implant designs. Tantalum beads are still needed for marking the bone segment.

The predictive value of RSA measurements for long-term performance of cementless implants is still elusive. There is a well-documented association between early migration of cemented implants and later loosening whereas this relationship is more complex in cementless implants (Kärrholm, 2012, Valstar et al., 2012). There is however a general agreement that RSA measured migration exceeding the detection limit is a good prognostic sign identifying implants at risk for later loosening, although the early migration tolerance threshold vary with implant design (Kärrholm, 2012). The high potential of RSA for early assessment of implant performance has been recognized by regulatory instances and RSA is suggested for screening of new implants before commercial introduction (Nelissen et al., 2011, Valstar et al., 2012).

RSA for monitoring osseointegration of cementless hip implants

For cemented femoral stems RSA studies have shown that early subsidence can predict later loosening and revision (Kärrholm et al., 1994). However, mechanisms of fixation is completely different for cementless stems and therefore the impact and predictive value of early migration or cut-off levels for revision risk cannot be adopted from cemented THAs.

RSA monitoring of hip implants have significantly increased understanding of the osseointegration process and clinical impact of micromotion. Early studies on cementless stems of different designs indicated that a 2-years postoperative subsidence exceeding 2 mm can predict later revision (Kärrholm et al., 1997). However, this has not been confirmed for newer stem designs. On the contrary, more recent short- and mid-term RSA studies on modern cementless designs indicate that early migration (up to 6 months) is acceptable, as long as stability is reached subsequently (Callary et al., 2012). With most cementless stem designs there seem to be a general pattern of early migration, mostly stem sinking, taking place during the first 3 to 6 months after implantation. Some designs show additional minor migration up to 1 year followed by stabilization. The magnitude and duration of initial migration of cementless stems differ depending on the design, surface topography, coating, and initial weight bearing (**Table 2.3**). Several cementless implant designs show migration > 1 mm during the first year, followed by fixation. It is suggested that migration during the second and third postoperative year can have greater predictive value in these cases (Kärrholm, 2012). The pattern and duration of migration is of greater value for predicting the long-term outcome of cementless THA than the numerical magnitude. The general migration pattern during the first two years allows for detection of implants at risk (Aspenberg et al., 2008, Callary et al., 2012).

Due to the limited number of long-term RSA studies, the predictive value of early migration on long-term survival of cementless femoral stems has not yet been confirmed. Mid-term (5-6 years) RSA follow-up of different stem designs have all demonstrated excellent clinical outcome and firmly fixed stems without additional migration regardless of magnitude of initial migration (3-6 months) in younger (mean age ≤60 years)(Ström et al., 2003, Wolf et al., 2010, Nysted et al., 2014, Nebergall et al., 2016) and older (60-80 years) patients (Callary et al., 2012, Weber et al., 2014, Skoldenberg et al., 2014, Flatoy et al., 2016). Ström and co-workers (2003) found that patients with significant stem migration at 2 years had stable and clinically functional stems at 5 years, even in a case with 1.75 mm subsidence. Similar results were reported by Wolf *et alia* (2010) studying the CLS stem in 38 patients (aged 25-65), demonstrating a 1.7 mm mean subsidence and 3.0 degrees mean rotation at 5 years. In five patients with subsidence between 3.5 and 6.4 mm at 1 year, stems had stabilized by 5 years with no further migration and clinically good outcome. Callary and co-workers (2012) studied the Corail stem in 27 patients (aged 55-80) and found that all stems had stabilized with good clinical performance at 6 years, regardless of early stem subsidence. At 6 months, one third of the patients had stem migration over 0.5 mm, and 17% had subsidence greater than 1.5 mm, with minimal further subsidence up to 2 years (Campbell et al., 2011). Between 2 and 6 years, no stem migrated more than 0.25 mm (Callary et al., 2012). In contrast to cemented THAs where 0.5-2 mm cut-off levels of initial motion have been set for identifying high risk revision implants, the usefulness of such threshold values for cementless femoral stems is unclear.

The impacts of bone bed quality and systemic bone condition on early stem migration have so far not been systematically evaluated. Despite extensive application of the RSA technology for monitoring cementless implant migration, it does not so far include patients with impaired bone quality or age-related geometrical changes of proximal femur. Most studies have been carried out on relatively young patients (age range <65 years), and mixed populations of male and female patients (**Table 2.3**). These populations do not represent the typical cementless THA patients, which based on national joint implant registers are females over 65. There is however a trend of higher mean age in later RSA-studies (**Table 2.3**).

TABLE 2.3 RSA studies on cementless femoral stems. Only marker-based studies included.

							Mean Subsidence (mm)			
Authors	**Year**	**Stem design**	**N (F/M)**	**Age (range)**	**Indication**	**Precision (mm)**	**2-6 months**	**12 months**	**24 months**	**Main findings**
Søballe *et al.,*	1993	Ti-coated	13	59 (50-68)	Not reported	0.1	2.5* (0.8)[e]	3.9* (0.8)[e]	Not measured	All stems migrated by 3 months. HA-coated subsided during 3 months, Ti-coated continued up to 12 months.
		HA-coated	15	57 (48-63)			1.9* (0.5)[e]	1.7* (0.4)[e]	Not measured	
Kärrholm *et al.,*	1994	Tifit HA	23 (12/11)	56 (38-63)	Primary OA Secondary OA (6)	0.25	0.04 (-0.9 to 0.2)[b]	0.07 (-1.2 to 0.8)[b]	0.04 (-1.2 to 0.8)[b]	Subsidence of the porous coated stem, only minor with the HA coated (p=0.02).
		Tifit porous coated	21 (13/8)	55 (45-63)			-0.1 (-0.7 to 0.2)[b]	-0.09 (-0.7 to 0.4)[b]	-0.1 (-2.7 to 0.3)[b]	
Kärrholm *et al.,*	2002	Epoch	38 (19/19)	58 (36-71)	Primary OA Secondary OA (3)	0.51	Not reported	Not reported	-0.02 (-0.16-1.04)[b]	Optimum fixation with both stems. Subsidence close to zero.
		Anatomic (zimmer)	30 (7/23)	61 (38-74)	Primary OA Secondary OA (2)		Not reported	Not reported	-0.05 (-0.31-0.31)[b]	
Ström *et al.,*	2003	Cone stem	13 (7/6)	42 (28-58)	Congenital dysplasia	0.29	0.3 (0.00-1.23)[b]	0.2 (0.02-0.69)[b]	0.3 (0.0-1.71)[b]	Migration within first 4 month. Cone stem showed good clinical outcome.
Grant *et al.,*	2005	Custom made	19 (21/16)	52 (31-65)	Hip OA	0.11-0.15	0.1	0.2	0.06	Marginal migration up to 2 year
Bottner *et al.,*	2005	ProxiLockHA Full weight-bearing	12 (1/11)	46 (35-59)	Hip OA	Not reported	-1.1 (-4.7-0.1)[b]	Not measured	Not measured	Difference in subsidence at 6 weeks. No difference at 6 months. Weight-bearing as tolerated recommended for young patients with excellent bone quality
		ProxiLockHA Partial weight-bearing	17 (4/13)	47 (24-59)	Hip OA		-0.4 (-1.7-0.4)[b]	Not measured	Not measured	
Luites *et al.,*	2006	ProxiLock	42 (27/15)	58 (35-77)	Primary OA Secondary OA (6)	0.2	-1.0 (-3.92 to 0.24)[b]	-1.1 (-3.63 to 0.32)[b]	-1.1 (-3.62 to 0.11)[b]	Migration pattern indicated insufficient primary fixation of ProxiLock in immediate weight-bearing. HA coating improved secondary stability. Implant use discontinued.
		ProxiLock HA					-0.8 (-3.29 to 0.12)[b]	-0.8 (-3.36 to 0.03)[b]	-0.9 (-3.32 to 0.00)[b]	
Ström *et al.,*	2006	Cone stem	22 (17/5)	55 (45-65)	Primary OA Secondary OA (5)	0.43	-0.5 (-2.49-0.07)[b]	-0.6 (-2.46-0.05)[b]	-0.5 (-2.51-0.03)[b]	Subsidence first 3 month, then stable. Excellent clinical 2 year results.
Ström *et al.,*	2006	CLS	29	55 (26-63)	Primary OA	0.24	-0.5 (-3.54-0.17)[b]	-0.5 (-3.58-0.34)[b]	Not measured	Small migration first 3 months. Thereafter stable. No effect of early weight-bearing.
Thien *et al.,*	2007	ABG I	43 (23/20)	53 (41-63)	Primary OA Secondary OA (3) Other (4)	Not reported	-0.3 (-4.3 to 0.16)[b]	-0.3 (-4.31 to 0.11)[b]	Not measured	No adverse effect of full weight bearing immediate after operation
Ström *et al.,*	2007	CLS	42 (20/22)	55 (<65)	Primary hip OA	0.24	-0.8 (-5.74 to 0.04)[b]	-1.2 (-6.37 to 0.03)[b]	-1.2 (-6.76 to 0.11)[b]	No difference in migration between partial and full weight bearing.
Simpson *et al.,*	2010	Furlong HAC	23 (14/9)	70 (33-87)	Hip OA	0.1	-0.3 (-0.51 to 0.03)[b]	-0.3 (-0.54 to -0.26)[b]	-0.4 (-0.55 to -0.24)[b]	More initial migration with the shorter stem (Active). Subsidence mainly up to 6 months.
		Furlong Active	20 (10/10)	71 (58-91)		0.1	-1.0 (-1.60 to -0.38)[b]	-1.1 (-1.59 to -0.55)[b]	-1.1 (-1.65 to -0.56)[b]	
Campbell *et al.,*	2011	Corail HA	27 (18/9)	70 (55-80)	Hip OA	0.04	-0.7 (-3.46 - 0.26)[b]	-0.6 (-3.66-0.35)[b]	-0.6 (-3.71-0.23[b])	Subsidence first 6 months. No further migration thereafter.
Baad-Hansen *et al.,*	2011	VerSys Fiber Metal Taper	41 (20/21)	60 (49-70)	Primary OA	0.2	-2.4 (-3.66 to -1.18)[a]	-2.3 (-2.98 to -0.64)[a]	-2.7 (-3.93 to -1.42)[a]	Major subsidence with both stems up to 2 years, predominantly up to 3 months. Patients need to be followed
		VerSys Fiber Metal MidCoat					-2.0 (-2.93 to -0.31)[a]	-1.8 (-2.88 to -0.77)[a]	-1.8 (-2.45 to -1.15)[a]	

*[a]95% CI; [b] Range; [c] Approximated from graphs; [d]median; [e]SEM; *maximum total point motion (MTPM); ±SD*

Continued TABLE 2.3 RSA studies on cementless femoral stems. Only marker-based studies included.

							Mean Subsidence (mm)			
Authors	**Year**	**Implant**	**N (F/M)**	**Age (range)**	**Indication**	**Precision (mm)**	**2-6 months**	**12 months**	**24 months**	**Main findings**
Bøe *et al.,*	2011	Taperloc HA	50 (31/19)	63 (27-81)	Primary OA	0.11	0.4[c]	0.3[c]	0.3±0.7	Stem subsidence first 3 month, then stabilized. No difference between groups.
		Taperloc BM					0.3[c]	0.4[c]	0.3±0.5	
Lindahl *et al.,*	2012	Taperloc HA	22 (16/6)	66 (64-68)	Primary OA	0.09	-0.5[c]	-0.4[c]	-0.5±0.82	Migration during first 6 months, thereafter stable.
Lazarinis *et al.,*	2013	CFP	27 (18/9)	56 (42-65)	Primary OA	0.09	-0.1 (-0.57 to 0.22)[a]	-0.1 (-0.94 to 0.35)[a]	-0.1 (-1.67 to 0.30)[a]	The CFP stem showed very little migration, characteristic of a stable implant
Weber *et al.,*	2014	Furlong HAC	25 (7/18)	63 (49-75)	Primary OA		-0.3 (-0.50 to -0.13)[a]	-0.3 (-0.52 to -0.14)[a]	-0.3 (-046 to -0.07)[a]	More 3 months subsidence with Active stem. Both stems stabilized after 3 months. No difference between the stems.
		Furlong active	25 (16/9)	62 (50-77)		0.08 mm	-1.0 (-1.64 to -0.35)[a]	-1.0 (-1.66 to -0.30)[a]	-1.0 (-1.66 to -0.29)[a]	
Edmondson *et al.,*	2014	K2	260	21-75	Diverse	Not reported	1.3 (0.35)[e]	1.9 (0.60)[e]	Not measured	Small subsidence, good clinical outcome. No difference between the stems.
		Apex modular					2.1 (0.57)[e]	2.5 (0.54)[e]	Not measured	
McCalden *et al.,*	2015	SMF (short)	22 (9/13)	63 (46-78)	Primary OA	Not reported	-0.6[c]	-0.8[c]	-0.9 (-5.86-0.03)[b]	No significant difference in stem migration at 2 years. Inconclusive study.
		Synergy	21 (12/9)	67 (45-81)			-0.3[c]	-0.3[c]	-0.3 (-1.57-0.28)[b]	
Salemyr *et al.,*	2015	Ultra-short HA Proxima	26 (15/11)	62 (±5)	Primary OA	0.54*	1.7*[d] (0.27-5.62)[b]	1.6*[d] (0.45-5.81)[b]	1.7*[d] (0.39-6.00)[b]	Migration first 3 month, then stabilized. Lower bone loss and equal excellent stem fixation with the ultrashort anatomical stem
		Bi-metric HA	25 (14/11)	62 (±6)			0.7* (0.08-7.05)[b]	0.6 (0.20-7.73)[b]	0.9* (0.87-7.47)[b]	
Flatoy *et al.,*	2016	Taperloc BM	49 (31/19	63 (27-81)	Not reported	0.11	0.1 (0.07 to 0.15)[a]	Not reported	Not reported	Migration first 3 months, not thereafter. No difference in migration or functional outcome compared to cemented stems.
		Taperloc HA					0.1 (0.06 to 0.14)[a]	Not reported	Not reported	
Sköldenberg *et al.,*	2011	Biomet Fracture stem Full HA	50 (36/14)	81 (79-92)	Femoral neck fracture	0.19	-0.2±0.5	-0.2±0.5	-0.2±0.5	Subsidence up to 3 months, no additional migration. Good stability and clinical outcome.
Figved *et al.,*	2012	Corail Full HA	11	78 (65-88)	Femoral neck fracture	0.02	0.1 (-0.02 to 0.29)[a]	0.2 (0.02 to 0.28)[a]	Not measured	Migration first 3 months, not thereafter. No difference in migration or functional outcome compared to cemented stems.
Schewelov *et al.,*	2012	Corail Full HA	38 (25/13)	81 (70-96)	Femoral neck fracture	0.1	-2.8[c] (0.8)[e]	-2.5[c] (0.6)[e]	-3.0[c] (0.8)[e]	Major migration of the fully HA-coated stem first 3 months. Good clinical outcome. No correlation between hip BMD and 2 yrs subsidence.

*[a]95% CI; [b] Range; [c] Approximated from graphs; [d]median; [e]SEM; *maximum total point motion (MTPM); ±SD*

2.6.5 Periprosthetic cortical bone remodeling

When a rigid metallic implant is placed into bone it alters the distribution patterns of physiological load. The bone remodeling response to the new mechanical conditions leads to accelerated bone resorption and net bone loss around the implant through phenomena referred to as adaptive remodeling and stress-shielding (Engh et al., 1987, Huiskes et al., 1989).

In adaptive remodeling, bone morphology adapts to changes in the mechanical environment. Under natural loading normal bone morphology is ensured through balanced remodeling. A femoral hip component causes unnatural load transfer, inducing morphological changes until a new equilibrium is reached. The significant changes in load distribution of the femur following THA reduce stress and strain and this unloading leads to disuse atrophy and bone loss according to Wolff's law (Weinans et al., 1992, Huiskes et al., 1989).

In the intact proximal femur greater compressive loads are transferred to the lesser trochanter, i.e. the calcar region, compared to the distal part. An implant shifts the load transfer from the proximal femur to the metadiaphyseal part, resulting in proximal unloading (Oh and Harris, 1978). The response is local resorption of the calcar region (calcar atrophy) and a varying degree of distal bone formation (hypertrophy). This phenomenon is known as adaptive bone remodeling (Engh et al., 1992). In addition, the shear stress at the implant-bone interface is increased due to mismatch in stiffness between the implant and the surrounding bone (Engh et al., 1987, Huiskes et al., 1989, Bobyn et al., 1992). In the femur, stress-shielding is caused by the high flexural stiffness of the implant material reducing the bending movements of the surrounding bone, causing increased unloading of the outer, cortical part of the bone (Engh et al., 1987, Huiskes et al., 1989). Stress-shielding seem to be the major factor causing periprosthetic bone loss (Tanzer et al., 2001, Skoldenberg et al., 2006). Disuse atrophy or adaptive remodeling following THA is typically seen in the proximal part of the femur, detected on radiographs as rounding-off of the calcar region. This is seen in all types of THA, more pronounced around bigger and stiffer stems (Ang et al., 1997). Bone loss due to stress-shielding is seen on radiographs as cortical thinning and diffuse decrease in periprosthetic BMD (Gruen et al., 1979).

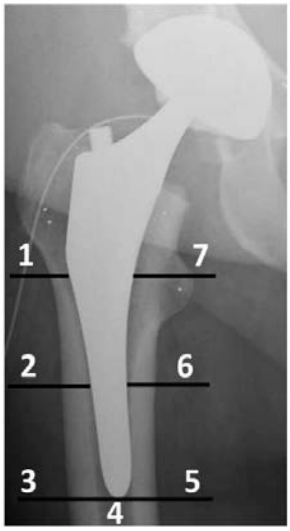

FIGURE 2.18 *Gruen zones used in DXA analysis of periprosthetic BMD.*

DXA evaluation of periprosthetic bone loss

DXA is the standard method for measuring and monitoring changes in periprosthetic BMD after THA. It is valuable for characterization of bone responses to implants, for evaluating new implant designs and for monitoring periprosthetic bone loss in risk groups, and as a readout tool in research. The method is sensitive, reproducible and accurate, and allows for detection of even small changes in bone mass (Cohen and Rushton, 1995, Kröger et al., 1996). The standard method is to measure BMD around the femoral implants in seven different regions of interest, also referred to as the Gruen zones (Gruen et al., 1979)(**Figure 2.18**). During DXA scanning, the patient's leg is positioned in a neutral rotation using a supporting device. Periprosthetic BMD is analyzed using software provided by the DXA manufacturer, which automatically removes the metallic implants on the scans and allows positioning of the seven regions. Results are presented as BMD (g/cm^2) and percentage change from baseline. There are several choices of baseline measurement. Measurement within 1 week postoperatively is to prefer over preoperative measurements or BMD of the contralateral hip (Venesmaa et al., 2001, Karachalios et al., 2007).

For controlling accuracy of periprosthetic measurements the DXA scanner is calibrated at manufacturing using a phantom, followed by weekly and daily phantom scans at the DXA facility to calibrate hardware and software parameters and radiation dose. Precision is determined by calculating CV between double examinations. Precision values typically are between 1-5% for the different Gruen zones, with average precision of 2-3% (Venesmaa et al., 2001, Lazarinis et al., 2013).

Clinical occurrence and consequences of periprosthetic bone loss

Periprosthetic bone loss occurs around both cemented and cementless THAs (Cohen and Rushton, 1995, Aldinger et al., 2003, Dan et al., 2006). Starting immediately after implantation the most dramatic decrease in BMD occur 3 to 6 months postoperatively, followed by a plateau at 12 to 24 months (Kiratli et al., 1996, Venesmaa et al., 2001, Aldinger et al., 2003, Wolf et al., 2010) with additional annual bone loss

corresponding to normal aging (Hannan et al., 2000, Venesmaa et al., 2003).

The magnitude and pattern of bone loss around cementless stems varies with the implant design, periprosthetic region and choice of baseline measurement. Total periprosthetic BMD has been reported to decrease by 5-10% during the first 2 postoperative years (Venesmaa et al., 2001, Grant et al., 2005, van der Wal et al., 2008, Boe et al., 2011b). The most substantial bone loss occur in the proximal femur, with 16-28% decrease in BMD during the first 2 years (Nishii et al., 1997, Venesmaa et al., 2001, Rahmy et al., 2004, Wolf et al., 2010, Lazarinis et al., 2013). While other areas start to recover after 1-2 years proximal bone loss is continuous, especially in female patient (Merle et al., 2011, Muren et al., 2015). Loss of proximal bone stock following THA is faster than normal age-related bone loss (Dan et al., 2006, Panisello et al., 2009b), and proceed for 4-10 years (Muren et al., 2015, Nysted et al., 2011, Aguilar Ezquerra et al., 2016) and even through the second decade after implantation (Merle et al., 2011).

The exact patterns and magnitude depends on location of stem fixation and thereby stress distribution to the surrounding bone (Aamodt et al., 2001, Khanuja et al., 2011). This is illustrated by the ABG stem, design to accommodate the natural geometry of the proximal femur to minimize stress-shielding (van Rietbergen and Huiskes, 2001, Van der Wal et al., 2006). Stem design modifications from distally fixation (ABG I) to more proximal fixation (ABG II) altered the remodeling patterns correspondingly, with more proximal bone preservation with AGB II (Van der Wal et al., 2006, Panisello et al., 2009a).

Large stem size (Nishii et al., 1997, Skoldenberg et al., 2006), high stem stiffness (Ang et al., 1997, Kärrholm et al., 2002), longer stems (Arno et al., 2012), proximal porous coating without HA (Tanzer et al., 2001) and extended porous coating (Yamaguchi et al., 2000) are factors associated with increased periprosthetic bone loss. Additional risk factors include increased age, female gender (Brodner et al., 2004, Merle et al., 2011), low local (Nishii et al., 1997) and systemic (Rahmy et al., 2004, Grochola et al., 2008, van der Wal et al., 2008) preoperative BMD. In OP and Dorr class C femurs reduced rigidity of the femur is associated with a need for larger stem sizes due to femoral canal widening. This increases the rigidity mismatch, leading to trochanteric bone loss and cortical hypertrophy around the tip. The clinical consequence is increased patient-reported thigh pain (Engh et al., 1987, Lavernia et al., 2004).

In cemented THA, extensive bone loss in the proximal femur predict later loosening (Nixon et al., 2007). In cementless THA long-term effects of periprosthetic bone loss are unclear. There are no evidences that severe bone loss causes immediate complications, or how proximal bone loss is related to long-term stem survival. Concerns are that continuous or massive bone loss may reduce the stability and cause challenges in case of revision surgery (Mayle and Paprosky, 2012). An earlier report demonstrated different patterns of bone loss around failed compared to successful THAs, regardless of fixation method or time since surgery (Venesmaa et al., 2000). Subjects with aseptic loosening have increased periprosthetic bone loss compared to subjects with fixed femoral stems (Wilkinson et al., 2003). Others have found no associations between stress-shielding and failure or revision (Engh et al., 2003). Some evidence suggests that loss of proximal bone, at least in some patient groups, increase the risk of periprosthetic fractures. Late-occurring periprosthetic fractures in cementless THA have been reported in retrospective larger cohort studies of mixed patients etiology (Hsieh et al., 2005, Streit et al., 2011) and in a prospective study of femoral neck fracture patients (Skoldenberg et al., 2014).

Increasing popularity of cementless THA in combination with more active patients and increased life expectancy, increase the probability of revision later in the patient's life. Therefore, conservation of periprosthetic bone is important. Postoperative antiresorptive drug therapy significantly prevent bone loss, especially in the proximal regions, as demonstrated with alendronate (Tapaninen et al., 2010), risedronate (Yamasaki et al., 2007, Skoldenberg et al., 2011a) and zoledronate (Scott et al., 2013). The bone preserving effect is seen around both cemented and cementless THA, with a stronger effect in cementless THA (Knusten et al., 2014). However, the effect persist only for 2 years and by 4-5 years periprosthetic bone loss is at the same level as untreated controls (Tapaninen et al., 2010, Muren et al., 2015). Further, there is no evidence that improved periprosthetic BMD improve implant function or decrease revision risk. Increased BMD through bisphosphonate therapy has not shown any effect on stem migration (Skoldenberg et al., 2011a, Friedl et al., 2009a), and stem designs with minimal migration do not seem to prevent from substantial proximal bone loss (Lazarinis et al., 2013). The current literature collectively indicates that no stem design can fully prevent proximal bone loss, but this does not seem to jeopardize primary stem stability or short-term outcome.

3 AIMS

The general aims of the studies were to investigate the impacts of preoperative bone quality and MSC capacity on the early migration and osseointegration of cementless stems in postmenopausal female patients. The specific aims were as follows:

I To delineate the frequency of low systemic BMD (osteoporosis or osteopenia) and other metabolic bone conditions in Finnish women scheduled for cementless total hip replacement for primary OA

II To optimize the osteogenic differentiation protocol of human bone marrow derived MSCs by adjusting the dexamethasone supplementation

III To investigate the impact of preoperative systemic BMD and other patient-related factors on periprosthetic cortical bone remodeling measured by DXA around the anatomically designed cementless femoral stem

IV To evaluate whether microstructural and mechanical properties of the local intertrochanteric cancellous can predict three-dimensional femoral stem migration and osseointegration determined by RSA

V To evaluate the effects of preoperative systemic BMD and age-related changes of proximal femur geometry on three-dimensional RSA-measured femoral stem migration and osseointegration

VI To investigate the in vitro osteogenic capacity of the bone marrow MSC reservoir in postmenopausal women undergoing cementless THA and how this correlates with RSA-measured femoral stem migration and osseointegration

4 HYPOTHESES

The general hypothesis of the studies was that successful and rapid fixation of the anatomically designed cementless femoral stem in postmenopausal women with primary hip OA is related to both the systemic and local bone quality, as well as to the osteogenic capacity of the patient's bone marrow MSCs. The specific hypotheses for the studies were as follows:

I Low systemic BMD (osteopenia or OP) and other undiagnosed metabolic bone disorders are more common than thought among women with primary hip OA scheduled to have cementless total hip replacement

II For *in vitro* osteogenic differentiation of human MSCs, transient treatment with 100 nM dexamethasone during the first week of induction culturing, as an alternative to continuous treatment, can provide enough stimuli for proper osteogenesis while negative effects of long term treatment could be overcome. With optimal conditions, variability can be reduced.

III Low systemic BMD (osteopenia or OP), alongside other patient-related factors regulating bone turnover, may interfere with the mechanical integrity of the anatomically-shaped femoral stem and thereby increase periprosthetic bone loss

IV Patients with decreased intertrochanteric cancellous bone quality will exhibit increased RSA-measured femoral stem migration and slower osseointegration compared to patients with normal cancellous bone quality

V Three-dimensional translational and rotational migration of the femoral stem is related to the systemic BMD status and the anatomical shape of the proximal femur, with increased migration in patients with low systemic BMD compared to patients with normal BMD

VI Individual variations in the in vitro osteogenic capacity of bone marrow MSCs will reflect in the process of implant healing. Patients with reduced osteogenic capacity of their MSCs will show increased femoral stem migration and delayed osseointegration

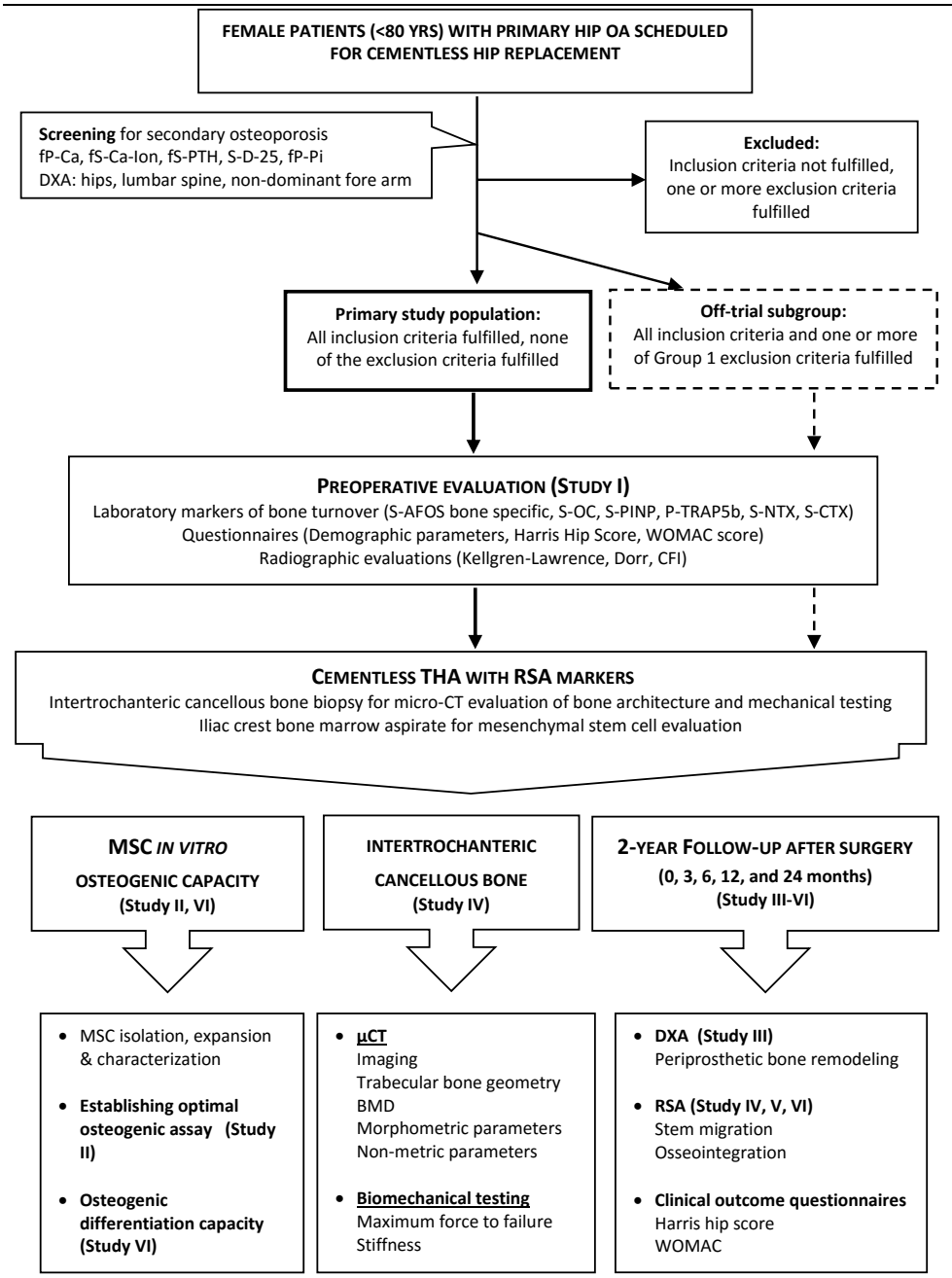

FIGURE 5.1 *Outline of the study protocol.*

5 PATIENTS AND STUDY DESIGN

*For this prospective two-year clinical trial investigating bone quality and its impact on cementless femoral stem healing in postmenopausal women, generally healthy patients less than 80 years of age with advanced primary hip OA scheduled for THA surgery at Turku University Hospital were considered. The specific inclusion and exclusion criteria were set to minimize confounding factors (**Box 9**).*

BOX 9. INCLUSION AND EXCLUSION CRITERIA

Inclusion criteria

- Female gender
- Degenerative hip OA as the indicator for cementless primary THA
- Age <80 years
- Signed informed consent

- Secondary OA of the hip
- Avascular necrosis of the femoral head
- Rheumatoid arthritis or other inflammatory arthritis
- Previous diagnosis of OP
- History of osteogenesis imperfecta or Paget's disease
- Severe OP (T-score < −3.5)
- Disorders of parathyroid function or D-vitamin metabolism (fS-PTH > 55 ng/l, S-D-25 < 22 nmol/l, fP-Ca > 2.47 mmol/l)
- Administration of drugs, which may interfere with bone metabolism, including:
  - Cumulative dose of 150 mg of prednisone or equivalent within the last 6 months
  - Calcitonin for 30 days or more within the last 6 months
  - Bisphosphonates for 30 days or more within the last 12 months
- Any other condition that, in the judgment of the investigator, would prohibit the patient from participating in the study
- Patient's refusal

5.1 PATIENT RECRUITMENT AND SCREENING

According to the standard of care at our institution at time of study initiation, cementless THA was performed for treatment of advanced primary hip OA if the patient was generally healthy, physically active and anticipated to resume an active lifestyle (most frequently ≤ 75 years of age), and routine X-rays did not show major abnormalities in bone structure of the proximal femur or pelvis. The study protocol is outlined in **Figure 5.1**.

Potential participants were identified from practice attendees and from the computerized record of the hospital district. An invitation to participate in the study was posted together with study details and a patient information sheet for written consent. From the 110 invitations sent, 70 positive responses were received (**Figure 5.2**). Based on preliminary examinations and interviews, two patients were excluded for not fulfilling the primary inclusion criteria and seven patients wanted to postpone the surgery. Hence, a total of 61 consecutive patients were recruited and underwent screening for eligibility and enrolment in the study. These 61 patients constituted the study base (**Table 5.1**).

Details of demographics, medications, and previous diagnoses and treatments were collected as part of the screening procedure. Patients were also screened for metabolic bone disorders by standard laboratory tests. Eight patients were excluded due to previously diagnosed OP (n=2) with antiresorptive bisphosphonate therapy, and peroral corticosteroid use (n=6) for rheumatoid arthritis, polymyalgia, erythema nodosum and Crohn's disease. Two of the patients received concomitant corticosteroid and antiresorptive bisphosphonate therapy (**Figure 5.1**). A total of 53 patients fulfilled the study criteria and were enrolled in the prospective trial (**Table 5.2**).

5.2 STUDY DESIGN AND FOLLOW-UP

The first step in the study was designed as a preceding step to the prospective two-year clinical trial and aimed to delineate the rate of undiagnosed primary and secondary OP among the 53 recruited patients (*I*).

In the prospective part of the study, clinical and radiological outcomes were evaluated at 3, 6, 12, and 24 months postoperatively. Based on screening, ten patients were excluded from the prospective trial because of severe undiagnosed primary OP (T-score < −3.5) requiring the initiation of antiresorptive drug therapy. Hence, 43 patients fulfilled the criteria for the prospective part of the study.

For the 2-year DXA follow-up of periprosthetic bone loss (*III*), two patients were excluded due to omitted baseline DXA measurements and two patients could not complete the study protocol due to surgical complications (periprosthetic fractures). Thus, 39 patients were included in these analyses.

An intertrochanteric bone biopsy was successfully obtained and analyzed from 35 of the 43 eligible patients (*IV*). For RSA measurements of stem migration and osseointegration (*V*), one additional patient was excluded due to surgical complications and one due to absence of RSA bone markers, leaving a total of 39 patients who completed the RSA-measurements. The basic patient characteristics for the subgroups of THA patients in studies I-VI are presented in **Table 5.2**.

During the screening process it became evident that the study would benefit from a parallel off-trial follow-up of excluded patients with severe undiagnosed primary OP requiring initiation of antiresorptive drug therapy (n=10), OP patients with ongoing antiresorptive drug therapy (n=2), and patients on corticosteroid use at baseline (n=4). Following accepted amendment by the Ethics Committee, the off-trial patients followed the same clinical and RSA study protocol.

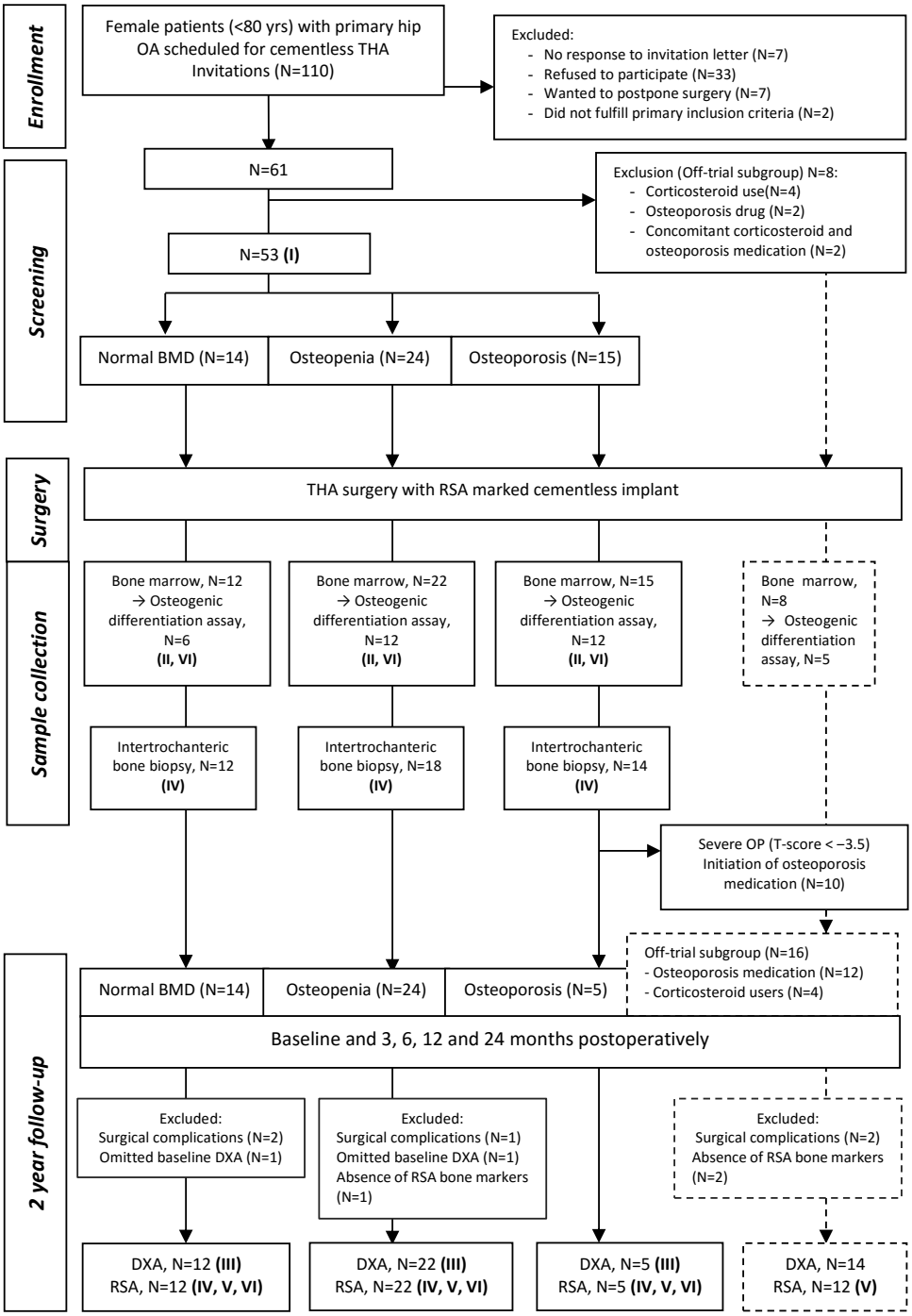

FIGURE *5.2* *Flow diagram of patient enrollment and follow-up*

5.3 EVALUATING MSC PROPERTIES

For studying the MSC properties of the THA patients, protocols for isolation, culturing and differentiation of bone marrow MSCs have been set up based on and modified from established protocols available at the time of initiation of the study. The osteogenic differentiation capacity was the main focus of the study, while other MSC properties served as part of the characterization protocol.

For setting up protocols for MSC analyses, and for comparison of the MSC capacity of THA patients, iliac crest bone marrow MSCs from a reference group of eight younger female fracture patients (mean age 37±17, range 19-60 years), without medication or diagnoses affecting the bone metabolism, were included (Table 5-3). The reference group was recruited as part of another study (Alm et al., 2010). The osteogenic differentiation assay of MSCs was further developed as part of the study (*II*), including MSCs from eleven THA females and six younger fracture females.

Of the 53 patients enrolled in the study, MSCs were successfully isolated, expanded and analyzed for osteogenic differentiation capacity from iliac crest bone marrow aspirates of 30 patients. Of these, 11 belonged to the group excluded from the 2-year RSA follow-up (severe OP n=9, surgical complications n=1, absence of RSA bone markers n=1). Consequently, 19 patients were included in comparisons of MSC capacity and RSA measured femoral stem migration (*VI*).

5.4 ETHICS

The study was conducted in accordance with the principles in the Declaration of Helsinki. The study protocols were approved by The Ethics Committee of the Hospital District of Southwest Finland, and all patients provided written informed consent prior to enrollment. The study was initiated, designed and performed as an academic investigation. Funding sources had no input or participation in conducting the study, analyzing the results or preparation of manuscripts.

TABLE 5.1 Preoperative patient characteristics of enrolled females with hip OA (n=61)

Age, mean±SD (range)	65±9 (41-79) years
BMI (kg/m^2), mean±SD (range)	30±6 (21-48)
Years since menopause	18±8 (range 1-33)
Estrogen replacement, n	11 (18%)
Use of calcium supplement	19 (31%)
Use of vitamin D supplement	18 (30%)
Smoking, n	5 (8%)
Alcohol consumption (drinks/week)	2 (range 0-10)
Vitamin D insufficiency (< 50 nmol/L), n	19 (31%)
Vitamin D < 80 nmol/L, n	48 (79%)
Previous fractures, n	23 (38%)
Preoperative medication, n	
OP medication	2 (3%)
Corticosteroid use	4 (7%)
Concurrent OP medication and corticosteroid use	2 (3%)
Systemic BMD (n, based on T-scores from 10 anatomical sites)	
Normal BMD	15 (25%)
Osteopenia	26 (43%)
Osteoporosis	20 (32%)
Initiation of OP medication (T-score < -3.5), n	10 (16%)
Disease scores	
Kellgren-Lawrence score	3±1 (1-4)
Harris Hip Score	50±15 (13-84)
WOMAC	50±16 (11-95)
Dorr classification (n)	
Type A	27 (44%)
Type B	26 (43%)
Type C	8 (13%)

TABLE 5.2 Preoperative patient characteristics of subgroups of THA females included in studies I-VI

Demographics	**Study I n=53**	**Study II n=11**	**Study III n=39**	**Study IV n=35**	**Study V n=39**	**Study VI n=19**
Age, mean±SD (range)	65±9 (41-79)	63±10 (41-75)	63±8 (41-79)	64±8 (41-78)	64±8 (41-78)	64±9 (50-78)
BMI (kg/m^2), mean±SD (range)	30±6 (21-48)	29±5 (21-39)	31±6 (21-48)	31±6 (21-48)	31±6 (21-48)	30±5 (23-39)
Postmenopausal, n	57 (93%)	10 (91%)	36 (92%)	32 (91%)	36 (92%)	19 (100%)
Years since menopause	18±8 (1-33)	17±6 (8-29)	17±8 (3-33)	18±8 (3-33)	18±8 (3-33)	19±8 (6-33)
Estrogen replacement, n	11 (18%)	2 (18%)	9 (23%)	8 (23%)	9 (23%)	5 (26%)
Use of calcium supplement	19 (31%)	2 (18%)	6 (15%)	6 (17%)	6 (15%)	1 (5%)
Use of vitamin D supplement	18 (30%)	2 (18%)	7 (18%)	7 (20%)	7 (18%)	1 (5%)
Vitamin D insufficiency (< 50 nmol/L), n	19 (31%)	4 (36%)	15 (39%)	15 (43%)	16 (41%)	9 (47%)
Smoking, n	5 (8%)	1 (9%)	4 (10%)	2 (6%)	3 (8%)	2 (11%)
Alcohol consumption (drinks/week)	2 (range 0-10)	2 (range 0-6)	2 (range 0-6)	2 (range 0-6)	2 (range 0-6)	2 (range 0-10)
Previous fractures, n	23 (38%)	4 (36%)	10 (26%)	7 (20%)	8 (21%)	5 (26%)
Systemic BMD						
Normal BMD, n	14 (26%)	2 (18%)	12 (31%)	12 (3%)	12 (31%)	4 (21%)
Osteopenia, n	24 (45%)	4 (36%)	22 (56%)	18 (51%)	22 (56%)	12 (63%)
Osteoporosis, n	15 (29%)	5 (46%)	5 (13%)	5 (14%)	5 (13%)	3 (16%)
Disease scores mean±SD (range)						
Harris Hip Score	49±15 (13-84)	50±16 (17-75)	49±15 (13-75)	51±16 (13-84)	50±16 (13-84)	53±16 (17-84)
WOMAC	51±15 (26-95)	55±15 (32-77)	51±16 (26-95)	49±15 (26-95)	50±16 (26-95)	50±15 (32-77)
Dorr classification (n)						
Type A	23 (43%)	8 (73%)	22 (56%)	20 (57%)	21 (54%)	11 (58%)
Type B	25 (47%)	2 (18%)	16 (41%)	15 (43%)	18 (46%)	8 (42%)
Type C	5 (10%)	1 (9%)	1 (3%)	0 (0%)	0 (0%)	0 (0%)

6 MATERIALS AND METHODS

For investigating systemic and local bone quality in female THA patients, the study covered preoperative baseline data including demographics and subjective data collected through questionnaires, radiological and laboratory evaluation of the patients' bone health, as well as the quality of intertrochanteric bone biopsies. The hip implant used was custom-modified for the study to allow for high-precision RSA measurements of 3D micromotions. The outcome of the cementless THA was assessed using a number of orthopaedic research methods. Clinical outcome was evaluated by patient satisfaction and hip specific outcome scores. Radiological outcome measures included traditional evaluation of plain radiographs, DXA analyses for assessing the extent of bone remodeling, and RSA of implant migration and stability. As an additional assessment of bone biology, this study investigated the osteogenic properties of the patient's bone marrow MSCs, and how this reflect in the osseointegration of cementless THA.

6.1 THE ABG II HIP IMPLANT

All patients received the cementless anatomically shaped femoral stem Anatomic Benoist Girard II (ABG II; Stryker Europe)(**Figure 6.1**). The stem is made of titanium alloy (TMZF®; Titanium, Molybdenum, Zirconium, Ferrous) with a low elastic modulus (Young's modulus 85GPa). It has a proximal HA coating (70 µm deep), aimed for proximal bonding and stress transfer. The distal part of the stem has been made short and undersized, with an ultrapolished surface to avoid distal bonding. The stem has a cervical-diaphyseal angle of 130 degrees, and a V40 taper.

The stem design is intended to re-establish physiological load transfer. The shape is aimed to follow the anatomical intraosseous contours of the proximal femur in all three dimensions, designed to achieve proximal press-fit in metaphyseal bone in order to ensure that the load transfer pattern imitates as closely as possible the natural distribution of bone stress within physiological limits (van Rietbergen and Huiskes, 2001). In the achievement of the best metaphyseal fill, the manufacturer's recommendation is to preserve the most cancellous bone but only if excellent intraoperative rotational stability is achieved. The stem is available in eight sizes. The stems used in the study were of size 3 (18 patients), size 4 (12 patients), and size 5 (13 patients). Ceramic heads (28 mm) and ceramic liners (both made of aluminum oxide ceramic; Al_2O_3) were used (ABG II; Stryker Europe). The acetabular component was a hemispherical cementless press-fit cup made of HA-coated titanium alloy (ABG II; Stryker Europe).

The ABG II stem was custom-modified for the study to allow for RSA measurements of micromotions. During manufacturing, the components were equipped with tantalum markers (**Figure 6.1**). The medial side and the tip of the stem were marked with a total of four RSA tantalum markers (1.0 mm in diameter). A peg containing two additional tantalum markers was inserted to the shoulder of the stem. Visualization of the tantalum markers was ensured through extensive studies using a phantom model. After appropriate mechanical testing, CE-marking of the device was applied in April 2003 and the prosthesis was available from June 2003. The manufacturer reconstructed and tested the prosthesis without charge and has not financially support this study. The hospital paid the normal price for the prosthesis.

6.2 SURGERY

The surgery was performed according to standard techniques using an anterolateral Hardinge approach. During surgery, 5-8 tantalum RSA markers (0.8 mm in diameter) were inserted into the greater and lesser trochanters of the proximal femur using the UmRSA® Injector™ (RSA Biomedical, Umeå, Sweden). According to the prevailing clinical practice, the patients were instructed to perform partial weight bearing; this was followed by full weight bearing after 6 weeks. At the time of surgery, a bone marrow aspirate was taken from the posterior iliac crest for MSC analyses, and an intertrochanteric bone biopsy was collected from the implantation site.

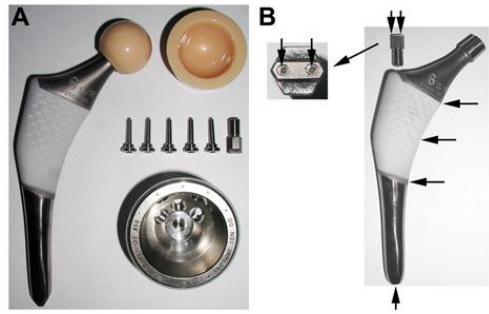

FIGURE 6.1. ***ABGII implant.*** *(A) The ABGII stem was used together with the ABGII acetabular component, along with ceramic heads and ceramic liners. (B) The implant was custom modified for RSA with tantalum beads (1.0 mm, arrows) at the medial side and the tip of the stem. A peg with two additional markers was inserted to the shoulder of the stem.*

6.3 LABORATORY TESTS

6.3.1 Standard laboratory tests

As part of the screening protocol, standard laboratory tests were obtained in order to detect metabolic bone disorders and vitamin D deficiency. Blood samples were collected between 8-9 a.m. after an overnight fast. Complete blood cell count, plasma calcium (P-Ca), serum ionized calcium (S-Ca-Ion), plasma phosphate (P-Pi), serum parathyroid hormone (S-PTH) and serum levels of 25-hydroxyvitamin D (S-25(OH)D) were measured. Values for ionized calcium were corrected to reflect the value expected at pH=7.4 (Ca^{2+}, 7.4). S-25(OH)D was measured using a radioimmunoassay (RIA)(25-hydroxyvitamin D Iodine 125 RIA, DiaSorin, Stillwater, MN, USA). S-PTH levels were measured using electrochemiluminescence immunoassay (Roche Diagnostics Ltd, Espoo, Finland). All tests were performed at the Turku University Hospital Laboratory according to standardized routines.

6.3.2 Serum markers of bone turnover

BLOOD SAMPLING

Biochemical markers of bone turnover were determined in order to evaluate the rate of bone formation and resorption. A 20 ml blood sample was collected between 8-9 a.m. after an overnight fast. Serum was isolated within 1h after collection by centrifugation at 2,000 x g for 10 min at room temperature, divided into 6 x 1.5 ml aliquots and stored at -70°C. Sample collection and serum isolation was performed at the Turku University Hospital Laboratory. Three serum markers of bone formation (OCN, ALP, PINP) and three serum markers of bone resorption (CTX, NTX, TRACP 5b) were analyzed. For each bone turnover marker, samples were analyzed in sets of 20 with appropriate duplicates and controls.

ANALYSES

Serum osteocalcin was measured using a RIA assay (OSTEO-RIACT, CIS, France). The OCN assay was performed according to standard protocol at Turku University Hospital Laboratory.

Serum bone ALP was determined by agarose gel electrophoretic separation (Beckman Paragon isopal electrophoresis system, Beckman Coulter, Inc., Belgium) and quantified by densitometry at 595 nm (Helena EDC system, Helena Laboratories, TX, USA). ALP assay was performed according to standard protocol by United Laboratories Ltd, Helsinki, Finland. Sensitivity of the assay was 1 U/l with intra- and inter-assay CVs of 0.8% and 2.2%, respectively.

The intact procollagen type I N propeptide (intact PINP) was measured in serum using a RIA kit (Intact PINP RIA, Orion Diagnostica, Espoo, Finland), according to manufacturer's protocol. The sensitivity of the assay was 2 µg/l, with intra- and inter-assay CVs of 10.2% and 7.9%, respectively.

Serum levels of C-terminal crosslinking telopeptide of type I collagen (CTX) was measured using an ELISA kit (Serum CrossLaps, Nordic Bioscience Diagnostics A/S, Herlev, Denmark). The sensitivity of the assay is 0.020 ng/ml and the intra-assay and inter-assay CVs are 5.2% and 6.7%, respectively.

N-terminal crosslinking telopeptide of type I collagen (NTX) was measured by ELISA (Osteomark® Wampole Laboratories Inc., Princeton, NJ, USA). The sensitivity of the assay is 3.2–40 nM, with intra- and inter-assay CVs of 4.6% and 6.9%, respectively.

Tartrate-resistant acid phosphatase type 5b (TRACP 5b) was measured in serum using an in-house ELISA (Halleen et al., 2000), with intra- and inter-assay CVs of 1.8% and 2.2%, respectively.

UNCOUPLING INDEX (UI)

As a measure for evaluating the balance of bone turnover, the uncoupling index (UI) was calculated (Eastell et al., 1993), taking into account all 6 markers of bone formation and resorption. The mean and SD from of the total data for each marker in all patients was determined. From these values, Z-scores ([Subject value – $Mean_{total}$]/SD_{total}) were calculated for each subject. The UI was calculated as the average of the Z-scores for the three bone formation markers minus the Z-scores for the resorption markers:

$$UI = \left(\frac{Z\ OCN + Z\ ALP + Z\ PINP}{3}\right) - \left(\frac{Z\ CTX + Z\ NTX + Z\ TRACP\ 5b}{3}\right)$$

A negative UI indicates that bone remodeling is unbalanced in favor of resorption, while positive UI indicates an imbalance favoring formation.

6.4 CLINICAL OUTCOME QUESTIONNAIRES

The Harris hip score was used as one tool for follow-up of subjective clinical outcome. It was used as self-reporting questionnaire that the patients filled in pre operatively (baseline score) and at 3, 6, 12 and 24 months after THA. At every visit, the pre-filled forms were gone through together with the patient in order to avoid misunderstandings and missed answers, since self-reporting-versions of the questionnaire has been criticized as less valid.

WOMAC score was used for evaluating the hip OA before (preop) and after (3, 6, 12 and 24 months post op) the replacement surgery. The patients filled in the self-reporting questionnaire prior to each visit, and the answers were gone through together with a health professional at time for the visit.

6.5 RADIOLOGICAL METHODS

6.5.1 Pre-operative radiological classification methods

The OA of the hip joint was assessed on weight-bearing antero-posterior radiographs of the pelvis.

KELLGREN-LAWRENCE GRADING

Classification of radiologic OA was performed by two independent observers using Kellgren-Lawrence grading system. If the scores differed by ≥ 2 points or a radiograph was scored as 1 by one reader and as ≥ 2 by the other reader, a consensus was agreed upon.

DORR CLASSIFICATION

As a qualitative measure of the shape and bone quality of the proximal femur, preoperative hip radiographs were used for classification of patients into the three distinct pattern types: Type A (normal), B, or C (straight/stovepipe)(Dorr et al., 1993).

CANAL FLARE INDEX (CFI)

As a quantitative measurement of morphological changes of the proximal femur the canal flare index was calculated (Noble et al., 1988).

In this thesis, the CFI was calculated from digital antero-posterior radiographs of the OA hips. Using an image analysis program, the most prominent point of the lesser trochanter was automatically traced, and the metaphyseal width 20 mm proximal to this point was easily measured (**Figure 6.2**). The intramedullary femoral isthmus width (G) was selected in a similar manner. The calculated CFI was used for classification of the canal shape of the femurs into normal (CFI 3-4.7), stovepipe (CFI <3) and champagne-flute (CFI >4.7).

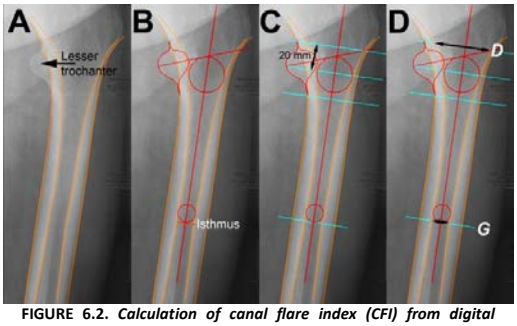

FIGURE 6.2. ***Calculation of canal flare index (CFI) from digital radiographs using an image analysis software.*** *(A) Preoperative digital radiographs of the OA-affected hips were imported to Rhinoceros CAD program. The software was applied to automatically trace the landmarks needed for calculation of CFI. (B) After tracing the cortexes, the most prominent point of the lesser trochanter and the intramedullary femoral isthmus were traced. (C-D) Thereafter the metaphyseal width 20 mm proximal to the most prominent point of the lesser trochanter,* D, *and the width at the isthmus,* G, *could be measured. CFI is the ratio of* D *to* G.

6.5.2 DXA measurements

6.5.2.1 Screening DXA and assessment of systemic BMD

Bone mineral content (BMC) and bone mineral density (BMD) were measured with Hologic QDR 4500C densitometer (Hologic Inc., Waltham, MA, USA) at the lumbar spine (from L1 to L4), both proximal femurs, and the distal non-dominant forearm. The quality control was maintained with daily measurements of a phantom provided by the manufacturer.

In 14 patients with previous unilateral THA, DXA of the contralateral proximal femur could not be measured. If the patient had suffered a fracture in the non-dominant forearm, BMD was measured from the dominant forearm. Using semi-automated positioning of regions of interests, the standard parameters of DXA scanning, including area (cm^2), BMC (g) and BMD (g/cm^2), were recorded. Bone density measurements were also expressed as T- and Z- score values that compare individual results to the normative database provided by the manufacturer (NHANES II).

The femoral neck regions of interest included a standard 15 mm of the femoral neck with its lower lateral border anchored at the base of the neck. This area was used to compare the size of the femoral necks in patients with bilateral DXA scans (***study I***).

ASSESSMENT OF SYSTEMIC BMD

For assessment of preoperative systemic BMD of the THA patients, DXA measured T-scores from all anatomical locations were included for classification as normal BMD, osteopenia or OP based on the WHO cut-off levels. Classification was done based on the lowest T-score of any lumbar vertebrae, distal forearm, femoral necks and total proximal femurs (a total of 10 measure sites) were used. A patient was classified as osteopenic if she had a T-score between −1 and −2.5 in any of the aforementioned sites. Correspondingly, a patient with a T-score less than −2.5 was classified as osteoporotic. Patients with a T-score < −3.5 at any given site were classified as having severe OP and were initiated on antiresorptive therapy, hence excluded from the prospective follow-up analyses. Z-scores, i.e., the standard deviation from the mean value of age-matched normal women, were utilized to explore whether patients with hip OA had different BMD compared to reference population of the manufacturer's database.

6.5.2.2 Periprosthetic DXA

For monitoring the extent of periprosthetic bone remodeling after THA, BMD around the femoral implant was measured at 7 days post-operatively (baseline) and subsequently at 3, 6, 12 and 24 months. During successive scans the femur was kept fixed in a neutral rotation using a leg positioning

device. Data analysis was performed using a software developed by the manufacturer (Metal Removal Option, Hologic), which automatically removes the metallic implants on the scans and allows studying of periprosthetic BMD by manual positioning of regions of interests. Periprosthetic BMD was determined from seven regions of interest (zones 1 to through 7) (Gruen et al., 1979). In addition, zones 1 through 7 were combined to form a total periprosthetic regions of interest. Changes in BMD over time were calculated using the immediate postoperative BMD as baseline and are expressed as percentage.

PRECISION OF PERIPROSTHETIC DXA MEASUREMENTS

To estimate the precision error of the Hologic DXA system, a total of 59 double scans were performed at different follow-up time points (baseline n=4, 3 months n=29, 6 months n=17, 12 months n=9). The patients were repositioned between the scans. The precision error, calculated as CV, varied from 1.5% to 3.4% depending on the region of interest, with an average of 2.3% which was comparable to precision errors in other studies (Kiratli et al., 1996, Venesmaa et al., 2001, Lazarinis et al., 2013).

6.5.3 Radiostereometric analysis (RSA)

RSA imaging

RSA examination was performed within seven days after surgery (baseline) and repeated at 3, 6, 12 and 24 months after surgery. A uniplanar set up was used, with the X-ray tubes positioned at 40° angle to each other in such a way that the X-ray beams cross each other at the site of the hip implant. The uniplanar calibration cage (Cage 43, RSA BioMedical Innovations AB) was placed under the examination table at a fixed height. The film–focus distance was set at 165 cm and the X-ray tubes were operated simultaneously in order to obtain paired images (stereoradiographs).

Standardization of the set-up including positioning of the two X-ray tubes and the calibration cage under the examination table, as well as visualization of the markers was ensured with preclinical studies of THA phantom models (Mäkinen et al., 2004). Image analyses were performed according to the RSA guidelines (Valstar et al., 2005).

RSA measurements

STEM MIGRATION COMPARED TO BASELINE

At each time point, migration of the stem in terms of three-dimensional rotation and translation were determined in relation to the postoperative baseline examination using the UmRSA 6.0.3.7 software (RSA BioMedical Innovations AB, Umeå, Sweden). The linear movements of the stem were analyzed as translations along three axes (x, y, z) and the angular movement was analyzed as rotations around three axes (x, y, z). In addition, the translation vector and the rotation vector were calculated as described previously (Kaptein et al., 2007). These traditional RSA measurements describe the change in implant position at each follow-up time point compared to baseline.

Based on a clinical point of view, the selected main parameters were translational migration along the y-axis (y-translation; stem subsidence) and rotational migration around the y-axis (y-rotation; anteversion-retroversion) as well as the translation vector and the rotation vector as indicators of overall 3-D micromotion.

QUALITY CONTROL AND PRECISION OF RSA SET-UP

The stability and adequate distribution of markers were assessed by calculating ME and CN (Valstar et al., 2005). The upper limit of mean error of rigid body fitting was kept around 0.35 and the upper limit of condition number at 150. In addition to the tantalum markers in the stem, the center of the femoral head was used as one marker. This allowed for better scatter of the stem segment and increased the reliability of the measurements.

Precision of the RSA measurements was determined by double examinations in all patients, with the patients repositioned between examinations. The mean value of the differences between double examinations plus two standard deviations represented the total error of determinations at the 95 per cent level of significance, based on normal distribution of each type of movement (Kärrholm et al., 1997, Bragdon et al., 2002). Thus, the precision of the selected RSA parameters was 0.42 mm for y translation, 0.40 for the translation vector, 1.81 degrees for y rotation and 1.32 degrees for the rotation vector.

When precision was calculated from those double examinations where the RSA markers on the medial side of the stem were not visible and the femoral head was used as the additional marker, precision values improved (0.17 mm for y translation and 1.20 degrees for y rotation), corresponding to those reported by other users of the UmRSA system.

CUMULATIVE STEM MIGRATION OVER TIME

Besides the traditional RSA measurements of change in implant position compared to baseline, cumulative migration detected between follow-up time points (3 to 6 months + 6 to 12 months + 12 to 24 months) was calculated as a measure of total distance of migration (***Study VI***). Since most cementless stem designs display some degree of migration, mostly subsidence, during the first 3 months settling period, we chose cumulative migration from 3 to 24 months as a measure to discriminate between normal expected migration and additional migration that can be more clinically relevant.

ASSESSMENT OF OSSEOINTEGRATION

In addition to the magnitude of stem migration, RSA data was further applied to assess stem osseointegration (i.e. cessation of migration as sign of implant healing and stabilization) at the individual patient level. At each time point, stems were classified as fixed (osseointegrated) or migrating by comparing to stem position at the preceding time point. The stem was defined as migrating if change in stem position exceeded the precision values of the RSA variable (0.42 mm for y translation, 0.40 for translation vector, 1.81 degrees for y rotation and 1.32 degrees for the rotation vector). Detection of cessation of translational and rotational migration was used to define the follow-up time point when each patient was found to have a stable osseointegrated stem.

Normal osseointegration was defined as stems that had stabilized by 3 months (i.e. following the settling period). Delayed osseointegration was defined as stem migration up to 6 months. Correspondingly, stems that migrated up to 12 months were designated as late osseointegration. Stems that showed continuous migration between 12 and 24 months were classified as unstable.

6.6 QUALITY OF INTERTROCHANTERIC CANCELLOUS BONE

6.6.1 Intertrochanteric bone biopsy

During surgery, a cancellous bone biopsy was taken from the proximal femur using a bone extractor instrument corresponding to the size of the implant. Biopsies were stored frozen at -20°C. Three cylindrical specimens (Ø 6 mm, thickness 5 mm) were prepared from each frozen biopsy using a high-speed trephine drill under saline irrigation to minimize fracturing of trabeculae (Hu-Friedy Mfg. Co., Inc. Chicago, IL, USA).

6.6.2 µCT imaging and microstructural analyses

The cylindrical specimens were scanned with a micro-CT under standardized imaging conditions (SkyScan 1072, SkyScan, Kontich, Belgium) with voxel resolution of 16.3 µm. This resolution is sufficient to produce accurate measurements of bone trabeculae (Salmon 2005). Three-dimensional analysis of trabecular bone geometry and BMD was performed with CTAn-software (SkyScan) using calibration with two phantoms (250 mg/cm^3 and 750 mg/cm^3) provided by the manufacturer. BMD could be measured by fitting Gaussian curves to the greyscale histograms using Origin software (Origin Lab Corp. Northampton, MA, USA). This method was also applied to calculate global threshold values for segment data sets for 3D analysis.

The analysis software provided calculations of morphometric parameters including bone volume fraction (BV/TV), mean trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N). Non-metric parameters, such as trabecular bone pattern factor (Tb.Pf), structure model index (SMI), and degree of anisotropy (DA), were also calculated.

6.6.3 Biomechanical testing

Following micro-CT imaging, the bone specimens were subjected to a destructive compression test using a universal mechanical testing device (Avalon Technologies, Rochester, MI, USA). Compression was performed in the A-P direction and a custom-made spherical socket was used to compensate for possible non-parallelism of specimen surfaces. The specimen-plate contact areas were oil-lubricated to reduce friction. Testing was performed at a constant speed of 0.825 mm/min and the load data was continuously recorded by a data acquisition system (Visual Designer, Intelligent Instrumentation, Tucson, AZ, USA). Maximum force to failure (ultimate strength) and stiffness were calculated from the raw data files using Origin software.

6.7 ANALYSES OF MSCs

6.7.1 Isolation and expansion of MSCs

6.7.1.1 Bone marrow aspirate

An iliac crest bone marrow sample of 3-5 ml was collected from each patient according to a modified protocol (Muschler et al., 1997). Briefly, bone marrow was aspirated from the posterior iliac crest under spinal anesthesia prior to surgery using a standard aspiration needle, and collected into a 10 ml syringe containing 1 ml of 1000 U/ml of heparin (monoparin®). The bone marrow was then mixed with 15 ml alpha modified essential medium (αMEM) containing penicillin-streptomycin (Gibco, Paisley, U.K) and 20 U/ml heparin (Heparin Leo; LEO Pharma A/S, Ballerup, Denmark) in a 50 ml tube and transported in room temperature to the laboratory for processing.

6.7.1.2 Isolation and culture expansion

Mononuclear cells (MNCs) were isolated from the bone marrow aspirate by density gradient centrifugation (Ficoll-Paque™ PLUS, Amersham Biosciences; density 1.077g/cm3). MNCs were collected after centrifugation at 400 x g for 30 min at room temperature, and seeded at 80,000 cells/cm^2. Cells were cultured in phenol red free αMEM with L-glutamine, ribonucleosides and deoxyribonucleosides, supplemented with penicillin-streptomycin and 10% pre-tested fetal calf serum that supports MSC growth (FCS; Gibco Invitrogen cat.#16000), hereafter referred to as basal medium or MSC-medium. Non-adherent cells were discarded after 48 h and half of the medium was changed twice

weekly. When cultures reached 70-80% confluence (approximately 14-21 days), cells were detached with trypsin (0.05% v/v in 1nM EDTA; Gibco Invitrogen) and re-seeded at 1000 cells/cm^2 (passage 0). Cells were re-seeded at the same density every 14-21 day for expansion. Cells from 12 patients were used for optimizing the protocol for MSC isolation and expansion by seeding cells at different densities.

6.7.2 Characterization of MSCs

MSCs were monitored for morphology and colony formation capacity, as well as proliferative capacity and growth kinetics through several passages. As part of the ISCT minimal criteria for identifying MSCs (Dominici et al., 2006), the MSC phenotype (CD105$^+$, CD73$^+$, CD90$^+$, CD45$^-$ and CD14$^-$), and chondrogenic differentiation capacity was confirmed for MSCs from a representative subgroup of patients by immunocytochemistry and micromass cultures, respectively. MSCs from all patients were analyzed for adipogenic and osteogenic differentiation capacity. In addition to the methods described here for detection of trilineage differentiation, expression levels of lineage specific mRNAs have been confirmed by real-time polymerase chain reaction as part of another study (Laine et al., 2012).

6.7.2.1 Morphology

The morphology of adherent cells was routinely monitored by light microscopy and documented by microphotographs.

6.7.2.2 Colony formation assay (CFU-F)

For analysis of CFU-F in primary bone marrow samples approximately 20,000 MNCs/cm^2 were plated in a 6-well plate (200,000 cells/well) and cultured in basal medium for 14-21 days. This assay was performed only when the total sample size allowed for it (primary culturing in flasks was prioritized). Preservation of colony formation capacity through culture expansion is considered a primary characteristic of MSCs (Sekiya et al., 2002). For monitoring the preservation of CFU capacity after each passage, cells were seeded at 10 cells/cm^2 in a 6-well plate (100 cells/well) and cultured in basal medium for 14-21 days. For evaluation, cells were fixed in 3% paraformaldehyde (PFA) for 20 min at room temperature and stained with crystal violet staining solution. Colonies were examined with light microscopy and total colony numbers were counted and presented as mean of triplicate wells.

6.7.2.3 MSC phenotype

As described in details elsewhere (Alm et al 2010), MSC phenotype was confirmed by immunocytochemistry from a subset of patients. MSCs were plated on glass cover slips at 2,000 cells/cm^2 and cultured for 1-2 weeks in basal medium. Thereafter cells were fixed in 3% PFA and immunostained for three positive (CD105, CD73, CD90) and two negative (CD45, CD14) MSC surface markers. Intrinsic peroxidase activity and non-specific binding were blocked by incubation with 3% H_2O_2 for 10 min, followed by a blocking with 3% bovine serum albumin for 30 min. The samples were incubated overnight at +4 °C with monoclonal primary antibodies against CD105 (SH-2, non-diluted hybridoma medium), CD73 (SH-4, 1:10) (kind gifts from A.I. Caplan, Case Western Reserve University, Cleveland, OH, USA), CD90 (ab23894, 1:10), CD45 (ab33533, 1:500) and CD14 (ab63319, 1:50) (Abcam). After washing, secondary biotin-conjugated anti-mouse antibody (E0354, 1:200, Dako) was added and incubated for 1 h. Bound antibodies were detected with avidin-conjugated peroxidase (1 h) and DAB (3,3'-diaminobenzidine, 2 min)(Vector Laboratories). Omission of primary antibodies was used as negative control. As positive controls for CD45 and CD14, human blood smears and isolated monocytes were used, respectively.

6.7.2.4 Proliferative capacity and growth kinetics

The proliferative capacity of MSCs was evaluated by calculating the number of cell population doublings (PDs) at each passage, using the formula *log N/log 2*, where *N* is the number of cells yielded at trypsinization of sub-confluent cultures divided by the number of cells seeded. Growth kinetics through several passages was expressed by calculating cumulative PDs. Results are presented as maximum PDs (max PD) i.e., the highest PD reach at a single passage (P0-3), and maximum PD rate (max PD rate) i.e., the maximum PDs/day at a single passage (P0-3).

6.7.2.5 Adipogenic differentiation

For adipogenic differentiation of MSCs, a culture protocol was modified from two protocols (Pittenger et al., 1999, Caterson et al., 2002). Passage 2-3 MSCs from all patients were seeded at 2500 cells/cm^2 in basal medium. After 3-4 days, media was changed to αMEM supplemented with 100 U/ml penicillin-streptomycin, 10% FCS (Gibco Invitrogen cat.#10270-106), 0.5 mM methyl-isobutylxanthine, 10 µg/ml insulin, 100 mM indomethacin and 1 µM dexamethasone (all from Sigma-Aldrich). When comparing protocols, no additional adipogenic differentiation was detected when switching between *adipose induction media* and *adipose maintenance media* as described in the literature. Therefore cells were cultured in adipogenic induction media only. Half of the culture medium was replaced every 3 or 4 day. Cultures in basal medium was used as a negative control. After 3 weeks, cells were fixed and adipocytic differentiation was detected by intracellular accumulation of lipid droplets and by staining for oil red O. Cells were washed with Ca^{2+}- and Mg^{2+}-free PBS and fixed in 3% PFA in PBS for 20 min at room temperature. Fixed cells were washed twice in

deionized water. Fresh Oil Red-O solution was prepared by mixing 3 parts of stock solution (0.5% in isopropanol) with 2 parts deionized water (3:2) and filter through a 0.2 μm filter. Fixed and washed cells were stained with oil red O solution for 2 h at room temperature, and washed with deionized water.

6.7.2.6 Chondrogenic differentiation

Chondrogenic differentiation assay was performed on passage 2 cells from a total of 12 THA patients and 6 patients from the reference group as standardized pellet cultures (Johnstone et al., 1998). Briefly 200,000 cells were washed in 15 ml conical tubes (BD Falcon) in 1 ml incomplete chondrogenic medium consisting of high glucose DMEM containing 4.5 g/l glucose (DMEM-HG) supplemented with 110 mg/l sodium pyruvate (Gibco), 50 μg/ml L-ascorbic acid-2-phosphate (Sigma-Aldrich), 100 nM dexamethasone (Sigma-Aldrich), 10 μl/ml ITS+ Premix (BD Bioscience), and penicillin-streptomycin (Gibco). Cells were re-suspended in complete chondrogenic media, comprising incomplete chondrogenic media supplemented with 10 ng/ml recombinant human TGF-β3 (rhTGF-β3) (R&D Systems) and pelleted by centrifugation at 500xg for 8 min. Pellets were maintained in culture with 1 pellet/tube in 1 ml complete medium. Medium was changed every 3-4 days, and rhTGF-β3 was always freshly added to the medium at every medium change. Pellets were harvested at 21 days and fixed in 3 % phosphate buffered formalin for 2 hours at room temperature. Fixed pellets were dehydrated by treatment with a series of graded alcohols, cleared by xylene and xylene substitute, and infiltrated with paraffin. Paraffin sections (5 μm) were deparaffinized using xylene and rehydrated in alcohol. Sulfated proteoglycans were visualized by staining with 0.1% Toluidine blue (Merck, Darmstadt, Germany) for 4 minutes and examined under light microscopy.

6.7.3 Osteogenic differentiation

6.7.3.1 Optimizing the osteogenic differentiation protocol by transient Dex treatment (II)

In order to optimize the osteogenic assay of MSCs, the benefits of limited supplementation with Dex in standard osteogenic induction media was investigated by comparing transient and constant Dex treatment. The aim was further to minimize inter- and intraindividual variations between donors. The two most commonly used concentrations of Dex (10 and 100 nM) were compared. The basic medium for osteoblastic differentiation consisted of basal MSC-medium supplemented with 10 mM β-glycerophosphate (Merck) and 0.05 mM ascorbic acid-2-phosphate (Sigma-Aldrich), hereafter referred to as OB-medium. Passage 2-3 MSC were plated at 2500 cells/cm^2 and half of the medium was changed twice weekly.

TIMING OF DEX SUPPLEMENTATION

Passage 2 MSCs from two donors (age 41 and 55) were cultured in OB-medium supplemented with either 10 nM or 100 nM Dex for days 1-7, 15-28, 21-28 or 1-28. Cultures without Dex were used as controls. For each condition, eight parallel 48-plate wells were used for each donor. Osteoblastic differentiation and mineralization was evaluated after 28 days with staining for ALP and von Kossa, respectively.

TRANSIENT VERSUS CONTINUOUS DEX TREATMENT

Based on results from the Dex-timing experiment, transient treatment for the first week (days 1-7) and continuous treatment (days 1-35) were chosen for further investigation. The effects on proliferation, viability and osteogenic differentiation were investigated. Five conditions were compared:

1) OB-medium
2) OB-medium with 10 nM Dex for the first week
3) OB-medium with 100 nM Dex for the first week
4) OB-medium with 10 nM Dex
5) OB-medium with 100 nM Dex

MSC-medium was used as a negative control.

6.7.3.2 Osteogenic differentiation capacity of MSCs from THA patients (VI)

The goal was to perform osteogenic differentiation on MSCs from all patients at passage 1, 2 and 3 (or 2, 3 and 4), as long as the number of harvested cells at each passage allowed for it. Through this approach, the primary aim was to ensure osteoblastic differentiation data from at least one passage/patient. Passage 1 and 3 MSCs were seeded in 12 wells of 24-well plates: four wells with basal medium and eight wells with OB-medium (four ALP staining, for von Kossa staining). This was done in two parallel plates; 2 weeks, and 4-5 week time points. Passage 2 MSCs were plated in two additional plates for ALP-activity and calcium assays, respectively. MSCs were seeded at 2500 cells/cm^2 in 24-well plates and cultured in OB-medium with 100 nM Dex the first 7 days. Half of the medium was changed twice weekly.

6.7.3.3 Detection and evaluation of osteogenic differentiation (II, VI)

ALKALINE PHOSPHATASE ACTIVITY (ALP ASSAY)

Osteoblastic differentiation was quantified by measuring cellular ALP activity. MSCs were cultured in OB-medium were extracted in 200 μl/well of Triton buffer (50 mM Tris-HCl, 0.1% Triton X-100, 0.9% NaCl, pH 7.6). After three cycles of freez-thawing cell extracts from each well were put in two parallel wells of a 96-well plate and incubated with the substrate p-nitrophenylphosphate for 30 minutes at pH 10. The enzyme activity was determined by measuring the absorbance at 405 nm in a plate reader (Wallac 1420 Victor^2, PerkinElmer Life Sciences, Turku, Finland).

Total protein content was quantified from the same cell extracts using a protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA, USA) and BSA as a standard (Bradford assay). ALP activity is presented as units/g protein.

QUANTIFICATION OF DEPOSITED CALCIUM (CA ASSAY)

The degree of mineralization was analyzed by measurements of extracellular calcium as described by Gitleman (1967), according to an established protocol (Qu et al., 1998). Cultures used for calcium assay were carefully checked for cell viability by light microscopy to minimize the risk of false positive calcium concentrations caused by dead cells. Cultures were washed three times with Ca^{2+}- and Mg^{2+}-free PBS and incubated with 200 µl/well 0.6 M HCl over night at room temperature. Deposited calcium was quantified using a calcium kit (Roche Diagnostics Corporation, IN, USA) and measured spectrophotometrically at 570 nm in duplicates of each well. Absolute calcium content was determined from a standard curve made of 2 mmol/l Ca solution provided by the kit manufacturer.

ALP STAINING

OB differentiation was visualized by histochemical staining for ALP. Cell cultures were washed with Ca^{2+}- and Mg^{2+}-free PBS and fixed in 3% PFA in PBS for 20 min at RT. ALP staining was performed using a histochemical kit according to the manufacturer's protocol (Sigma Diagnostics, St. Louis, MO, USA). Naphtol-AS-TR-phosphate was diluted 1:5 in water. Fast Red TR was diluted 1:1.2 in 0.1 M Tris buffer (pH 9.0). The solutions were mixed 1:1 and added to the fixed cells as substrate for ALP. After incubation for 1 h at room temperature, ALP staining was detected.

VON KOSSA STAINING

Mineralization of extracellular matrix was visualized by the von Kossa silver staining method for phosphate deposition. Fixed cells were washed twice with deionized water and incubated with 2% silver nitrate (Sigma-Aldrich) for 1 h under direct light from a 60-W lamp. Excess silver nitrate was washed away by incubating in 2.5% sodium thiosulphate (Merck) for 5 min at room temperature and cells were subsequently rinsed three times with distilled water and allowed to air dry.

QUANTIFICATION OF STAINED AREAS

ALP and von Kossa stained areas were quantified by histomorphometric methods. Stained multiwell plates were scanned using a flatbed scanner with a transparency adaptor (HP ScanJet 5370C) at 600 dpi resolution and saved as 24-bit color images in TIF-format. Transparency exposure adjustments were kept constant to create images of equal intensity. ALP and von Kossa positive areas were quantified histomorphometrically from the images using an automated image analysis (Valimäki et al., 2006) and stained areas (cm^2) were converted to percentage of total culture area.

IMMUNOCYTOCHEMICAL STAINING FOR TYPE I COLLAGEN AND OSTEOCALCIN

Terminal OB differentiation was demonstrated by immunostaining for COL1 and OCN. MSCs from two donors were cultures in 4-chamber slides (Nunc) at 2500 cells/cm^2 and culture for 7, 14, 21, and 28 days. Cells were fixed and intrinsic peroxidase activity (3% H_2O_2, 10 min) and non-specific binding (3% BSA, 30 min) were blocked. Samples were incubated overnight at +4 °C with polyclonal primary antibodies against COL1 (1:100, Fitzgerald Industries International, Concord, MA, USA) or OCN (1:60, AbD Serotec, Oxford, UK) and then with a secondary biotin-conjugated anti-mouse antibody (1:200, Dako) for 1 h at room temperature. Bound antibodies were detected with avidin-conjugated peroxidase (1 h) and DAB (Vector Laboratories). Omission of primary antibodies was used as negative controls.

6.7.4 Assessment of cell proliferation, viability and apoptosis (II)

6.7.4.1 Cell proliferation (MTS-assay)

To assess the effect of Dex on cell proliferation, MSCs from four donors were seeded at 1000 cells/cm^2 in 96-well plates. Cell viability was determined at days 3, 7, 14 and 21 using an MTS-assay (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay, Promega Co., Wisconsin, USA) according to the manufacturer's protocol. The optical density at 490 nm (OD_{490}) was used as a cell viability index as described previously (Itälä et al., 2002). The MTS assay is a colorimetric method based on the conversion of MTS by dehydrogenase enzymes in metabolically active cells into a formazan product that is soluble in culture media and can be measured as absorbance at 490 nm. As such, the assay measures the number of living cells and can be applied to monitor changes in cell number as a measure of proliferation.

6.7.4.2 Cell viability (Live/Dead assay)

The effects of Dex on cell viability was studied by evaluating the amount of living and dead cells at different time points using a LIVE/DEAD kit (Molecular Probes, Eugene, OR, USA), which is based on the simultaneous detection of two probes. Live cells are detected by fluorescent green calcein (calcein AM) produced by intracellular esterase activity, while dead cells are detected by ethidium homodimer (EthD-1) that enters cells with damaged membranes and produces bright red fluorescence upon binding to nucleic acids. For the assay, MSCs were seeded at 1000 cells/cm2 in 96-well plates. Living and dead cells were quantified at days 3, 7, 10,

14, 21, 28 and 35. The assay was performed by incubating cells with 1 µM calcein AM and 1 µM EthD-1 in PBS for 20 minutes at +37 °C, 5% CO_2. The fluorescence counts at 530/642 nm (dead cells) and 485/535 nm (live cells) were measured with a 96-well plate reader (Wallac 1420 Victor2, PerkinElmer Life Sciences, Turku, Finland). Visualization of living and dead cells was achieved by staining with Calcein and EthD-1. MSCs cultured on coverslips were incubated with 2 µM calcein AM and 2 µM EthD-1 for 20 minutes in +37 °C, 5% CO_2, rinsed with PBS and immediately observed under fluorescence microscope.

6.7.4.3 Apoptosis assay (Cell death detection ELISA)

The effects of Dex on apoptosis was studied using a photometric ELISA assay (Cell death detection ELISA^{PLUS}, Roche Diagnostics) which detects cytoplasmic histone-associated DNA fragments. Briefly, MSCs were seeded at 2500 cells/cm^2 in 24-well plates and cultured for 3, 10, 14 or 21 days. The cells were lysed in 200 µl lysis buffer/well and lysates from three replicate wells were combined for the assay, which was performed in two replicates.

6.8 DATA ANALYSES AND STATISTICAL METHODS

6.8.1 Data handling and statistical strategies

For analyzing data from the different studies, statistical methods were chosen based on the clinical questions. Therefore, in addition to analyze raw data, many of the continuous variables describing patient characteristics (e.g., systemic BMD, bone turnover markers, vitamin D levels), as well as clinical follow-up variables (RSA measured osseointegration), were converted into clinically more relevant categorical variables. This also improved the statistical power by overcoming large patient variability in some continuous variables. Categorizing was done by dividing data into quartiles. Due to the relatively restricted number of patients, the two middle quartiles were usually combined to form a middle quartile.

Before performing statistical analyses, all continuous data were tested for normal distribution and equal variance using Kolmigorov-Smirnov test and Levene's test, respectively. Data fulfilling these assumptions were analyzed using parametrical statistics. In case data did not follow normal distribution and/or equal variances non-parametric equivalent tests were used. Statistical significance was defined as p-values less than 0.05.

Outliers

For analysis of continuous variables, possible outliers were identified as values lying outside 1.5 interquartile ranges from the first and the third quartiles. The influence of such outliers (extreme values) on the outcome and conclusion of statistical testing was checked by performing testing with and without the outliers. Especially in correlation and regression analysis, the influence of extreme values on the overall conclusions was carefully controlled. Outliers were identified in data of serum markers of bone turnover, in some of the parameters for assessing osteogenic differentiation capacity of MSCs, and in RSA data. Outliers were included when these continuous variables were converted into categorical variables.

6.8.2 Data analyses study wise

Study I

Demographic data and DXA values were normally distributed and are presented as mean values with standard deviations (SD). For comparison of patients with normal and reduced systemic BMD, ANOVA with the Holm-Sidak *post hoc* test was used for continuous variables, whereas the chi-square test was used for categorical variables. The Kellgren-Lawrence scores between the groups were analyzed by Kruskal-Wallis ANOVA on ranks.

Laboratory data and biochemical markers of bone turnover were not normally distributed and are presented as medians with 95% confidence intervals. They were analyzed by non-parametric Kruskal-Wallis ANOVA on ranks with Dunn's *post hoc* test. The relationships between T-scores and biochemical markers of bone turnover were analyzed using Spearman rank order correlation. Comparisons of DXA measurements between the OA affected hip and the contralateral hip was performed using paired T-test. All statistical analyses were performed using SigmaStat 3.0.1 software.

Study II

The effects of dexamethasone and different Dex treatments on osteogenic differentiation of MSCs were analyzed using mixed models analysis of variances, with culture medium as fixed factor and donor as random factor to allow for multiple replicates from each donor. The test accounted for the dependent nature of the data when cells from same donors were subjected to several treatments. In all mixed models analysis, Sidak's adjustment for multiple comparisons was used.

Influence of donor age was analyzed using both mixed models analyses and linear regression, with age as a cofactor in both analyses. To investigate if the same culture condition is optimal regardless of donor age, donors were divided into three age groups based on age periods of differing MSC titers (Caplan, 2004) and analyzed separately (Group I <30 years, Group II 30-60 years, Group III >60 years). To assess the magnitude of variation between donors, and between

parallel samples from the same donor, inter- and intra-individual CV (%) were calculated (SD/mean x 100). All statistical analyses were performed using SPSS 19.0 for Windows.

Study III

The main variable investigated, time-related BMD change in periprosthetic bone regions, was analyzed using repeated measures ANOVA with Bonferroni's correction for multiple comparisons between the time points.

The statistical significance of preoperative patient-related factors for prediction of time-related periprosthetic BMD changes was evaluated using ANOVA for repeated measurements (categorical factors) and analysis of covariance for repeated measurements (continuous factors) with Tukey's adjustment for multiple comparisons. The significance of each patient-related factor for the observed BMD changes at the endpoint was determined using linear models with calculations of the coefficients of determination (R^2) for these models. Continuous variables were treated as covariates, while categorical variables were treated as fixed factors with Tukey's adjustment for multiple comparisons when comparing the categories. Demographic patient-related factors investigated included age, BMI, serum vitamin D level, previous fractures, alcohol consumption, and preoperative WOMAC and Harris hip scores. Also the radiological parameters CFI, Dorr classification and Kellgren-Lawrence scores were analyzed for their value as predictors of periprosthetic BMD changes.

When investigating association between rate of bone turnover and changes in periprosthetic BMD, biochemical serum markers were analyzed by quartiles with the two middle quartiles combined (lowest 25 %, middle 50 %, highest 25 %). In addition, the uncoupling index was applied to evaluate the balance of bone turnover.

Association between preoperative BMD and periprosthetic bone loss in the femur was investigated using both the local BMD of the operated hip and the systemic BMD (based on the lowest T-scores). Differences in absolute BMD of Gruen zone 7 between the 3 patient groups (normal BMD, osteopenia, and OP) were analyzed at baseline and at 24 months using one-way analysis of variances with Tukey's adjustment for multiple comparisons. For Gruen zone 7, the association between baseline BMD and absolute loss in BMD (g/cm^2), as well as the association between change in BMD (%) and the lowest preoperative systemic T-score were analyzed with linear regressions and presented with the coefficient of determination (R^2).

Statistical analyses were done using SAS System for Windows, release 9.1 (SAS Institute Inc., Cary, NC, USA). Linear regression models were done using SPSS 16.0 for Windows.

Study IV

To investigate the effect of biomechanical and microstructural properties of intertrochanteric cancellous bone on RSA stem migration linear regression analysis was applied with migration expressed as absolute (unsigned) values. The predictive value of the biomechanical and microstructural parameters on time related stem migration was analyzed using repeated measures ANOVA with biomechanical and microstructural parameters as covariates. Logistic regression analysis was applied to estimate the effect of biomechanical and microstructural parameters on time point of osseointegration (cessation of translational and rotational migration).

Based on μCT measured BMD, patients were divided into low (25%), middle (50%) and high (25%) local BMD based on quartiles. Differences in the magnitude of stem migration were analyzed using one-way ANOVA with Tukey's *post hoc* test. Within the local BMD quartiles, the probability of normal, delayed, or late osseointegration, or the presence of an unstable stem was examined as Kaplan-Meier time-to-event estimates.

Linear regression analyses were further used to investigate the associations between 1) biomechanical and microstructural properties, and 2) demographic characteristics and biomechanical properties, as well as microstructural properties. All statistical analyses were performed with SPSS 16.0 (SPSS, Chicago, IL, USA).

Study V

The magnitude of femoral stem migration is presented as signed mean ± SD. Stem migration from baseline to 3 months was analyzed with paired t-test, while the overall migration during the 24 month study period was analyzed using repeated measures ANOVA. To investigate whether time related stem migration was different in patients with normal systemic BMD compared to patients with low systemic BMD (osteopenia and OP), group was used as cofactor in repeated measures analysis. Differences between the two groups were further investigated at each time point using Student's t-test and results are presented as mean difference with 95% confidence interval (CI) for the difference, along with p-values.

Differences in the time point of implant osseointegration (stable/unstable stem at 3, 6, 12 or 24 months) between the two study groups (normal systemic BMD versus low systemic BMD) were analyzed using Kaplan-Meier time-to-event estimates. The only censored observations were the unstable stems at 24 months.

The effect of demographic parameters on implant osseointegration was analyzed with a logistic-

regression model adjusted for age. Parameters investigated were age, BMI, local BMD and T-score of the operated hip, and canal flare index. Odds ratios with 95% CI and p-values were used to estimate the effect on stem stabilization.

Time related changes in WOMAC and Harris Hip scores were evaluated with repeated measures ANOVA. Difference in scores between the two study groups at each time point was investigated with Student's t-test and presented as mean differences with 95% CI and p-values.

The off-trial patients were analyzed as two groups: patients with bisphosphonate treatment and patients on corticosteroids. Time related stem migration in the off-trial patients was compared with the study patients using repeated measures ANOVA with group as cofactor. The magnitude of stem migration at 3 and 24 months in the two off-trial groups was compared to those of the two main study groups using Mann-Whitney U test.

One patient who showed an abrupt 8.5 degrees of rotation of the stem by 3 months followed by minimal further migration by two years was excluded from the statistical analysis as an outlier. For all repeated measurements, sphericity was checked with Mauchly's test. In cases where sphericity was not fulfilled, the Huynh-Feldt correction was used. All statistical analyses were performed with SPSS 19.0 (SPSS, Chicago, IL, USA).

Study VI

OSTEOGENIC CAPACITY GROUPS

For studying the osteogenic differentiation capacity of MSCs in THA patients in relation to femoral stem osseointegration, MSC data was successfully obtained from 30 patients. Although MSCs from all these donors demonstrated osteogenic differentiation, it was at varying degree. The level of mineralization was generally low, and complete mineralization was detectable only for MSCs from part of the patients. From 13 patients, no matrix mineralization could be detected despite repeated osteogenic culturing at two or three different passages (p1-3). These MSCs were hence classified as negative for mineralization. Due to the large interindividual variability in the MSC data, patients were classified as having MSCs with *high* or *low osteogenic (OB) capacity* based on the combined ALP and mineralization outcome for the purposes of analyzing the relationship between *in vitro* osteogenic differentiation capacity of MSCs and hip implant migration. The classification strategy included three steps for dividing the patients in an objective way based on both differentiation and mineralization capacity.

Step 1. Patients were ranked for osteogenic differentiation capacity, i.e. ALP stained area, by arranging the data in ascending order and dividing into 1) low, 2) middle and 3) high index groups of equal size. Osteogenic differentiation capacity index based on ALP stained area:

Low: 1-20% ALP stained area, N=6
Middle: 21-57% ALP stained area, N=7
High: 68-90% ALP stained area, N=6

Step 2. Patients were ranked for mineralization capacity of their MSCs, i.e., von Kossa stained area, using the same method. Mineralization capacity index based on von Kossa stained area:

Low: 0% von Kossa stained area, N=7
Middle: 5-16% von Kossa stained area, N=6
High: 18-91% von Kossa stained area, N=6

Step 3. The OB-capacity groups were obtained by calculating the sum of ALP and mineralization indexes for each patient. The low OB-capacity group was defined as patients with a combined index between 2 and 4, and the high OB-capacity group was defined as patients with a combined index of 5 and 6. This strategy ensured that the high OB-capacity group did not include any patient with low ranking in either of the MSC quantification methods,

TABLE 6.1 *Classification of patients based on combined ALP and mineralization index, and levels of ALP and von Kossa in the two OB-capacity groups.* * Student's T-test

Combined OB-capacity index	*N*	Group	ALP stained area (%) mean±SD (range)	Von Kossa stained area (%) mean±SD (range)
2	2	Low OB-capacity	35±27 (1-76)	5±9 (0-32)
3	6			
4	4			
5	5	High OB-capacity	62±25 (22-90)	27±17 (5-52)
6	2			
		p-value*	0.050	0.023

STATISTICAL ANALYSIS

Data is presented as mean ± SD (range). Comparisons between the low and high OB-capacity groups were done using Student's T-test. Possible influence of confounding demographic and clinical factors was ruled out by separate linear correlation analyses with the study main parameters (MSC data and RSA data, respectively). The relationship between *in vivo* bone quality parameters and *in vitro* osteogenic properties of the patients' MSCs (N=30) was analyzed with linear correlation and presented with Pearson correlation coefficient along with p-values. Prior to any correlation analyses, data was checked for normal distribution and outliers. Kaplan-Meier time-to-event estimates were applied to analyze differences in osseointegration time point. Statistical analyses were performed using IBM SPSS Statistics 22 software.

7 RESULTS

7.1 PREOPERATIVE FINDINGS (I)

7.1.1 High prevalence of undiagnosed osteopenia and OP in women with hip OA

The THA patients were classified as having normal systemic BMD, osteopenia or OP based on T-scores from lumbar spine, proximal femurs and distal non-dominant forearm. Only 14 of the 53 hip OA patients (26%) had normal systemic BMD. Osteopenia was found in 24 patients (45%) and 15 patients (28%) had previously undiagnosed OP (**Table 7.1**). Of the 24 osteopenic patients, ten had osteopenic values only in 1 or 2 of the anatomical sites measured, and normal values in the remaining 8-9 areas (**Table 7.2**). Seven of the patients had osteopenic values in more than five areas.

In the 15 patients classified as having OP, 11 had OP values only in 1 or 2 areas. None of the patients had osteoporotic values in more than five areas (**Table 7.2**). A total of 10 patients were diagnosed with severe OP and antiresorptive drug therapy was initiated. Eight patients had at least one area with a T-score less than −3.7 (range −3.70 to −5.50). The remaining five osteoporotic patients had only one area with T-score less than −2.5, except for one patient who had four osteoporotic values, but five areas with normal values.

Highest prevalence of osteoporotic values (<−2.5) were found in lumbar spine, representing 17 out of the total number of 32 osteoporotic values (ten measured areas in 15 patients), followed by the non-dominant forearm representing 8 out of the total number of 32 osteoporotic values. Normal values were predominantly in the OA affected hip, although two patients had osteoporotic values in their OA affected hip. The same pattern was found for the osteopenic values (<−1.0) in the osteopenic group. However, when looking at osteopenic values in all patients with low systemic BMD (osteopenia + OP), osteopenic values were found at similar frequency in all measured sites.

Osteoporotic patients were older (p=0.015) and had lower BMI (p=0.05) compared to patients with normal systemic BMD or osteopenia, respectively (**Table 7.1**). All osteoporotic and osteopenic patients were postmenopausal, while three patients with normal BMD were premenopausal. The use of estrogen replacement was more frequent in the normal BMD group (p=0.025).

TABLE 7.1 *Baseline patient characteristics obtained from screening (n=53).*

Demographics[a]	Normal BMD n=14	Osteopenia n=24	Osteoporosis n=15	*p-value*
Age, mean±SD (range)	60±10 (41 – 78)	65±6 (55 – 74)	68±9 (49 – 79)	0.018 *
BMI (kg/m^2), mean±SD (range)	30±7 (21 – 43)	32±6 (23 – 48)	28±4 (21 – 33)	0.066 #
Postmenopausal, n	11	24	15	0.012
Years since menopause	14±9 (5 – 28)	17±8 (2 – 33)	20±9 (3 – 31)	0.147
Estrogen replacement, n	6	3	1	0.025
Use of calcium supplement	4	4	4	0.635
Use of vitamin D supplement	3	4	5	0.477
Previous fractures, n	5	6	7	0.568
Laboratory findings (Median, 95% confidence interval)[b]				
P-Ca (mmol/l)	2.3 (2.2 to 2.5)	2.3 (2.1 to 2.5)	2.4 (2.2 to 2.6)	0.178
Ca^{2+}, pH 7.4 (mmol/l)	1.2 (1.2 to 1.3)	1.3 (1.2 to 1.3)	1.2 (1.2 to 1.3)	0.374
S-25(OH)D (nmol/l)	55 (28 to 93)	58 (30 to 97)	59 (33 to 89)	0.981
Vitamin D status				0.636
Adequate (>75 nmol/l)	5	5	2	
Insufficiency (50-75 nmol/l)	4	11	7	
Deficiency (<50 nmol/l)	5	8	6	
S-PTH (ng/l)	37 (18 to 72)	39 (21 to 74)	43 (19 to 97)	0.226
Elevated PTH, n	3	1	6	0.014
Endocrinologist consultation, n	1	1	6	0.006

[a] One-way ANOVA for continuous variables and chi-square test for categorical variables
[b] Kruskal-Wallis test for continuous variables and chi-square test for categorical variables
* Significant difference between patients with normal BMD and osteoporosis
\# Significant difference between patients with osteopenia and osteoporosis

TABLE 7.2 *Number of anatomic areas with low T-scores in patients classified as osteopenic and osteoporotic (10 areas measured).*

Number of affected areas	Osteopenia, n=24 (T-score between -1 and -2.5)	Osteoporosis, n=15 (T-score < -2.5)
1 – 2	10	11
3 – 5	7	4
> 5*	7	0

* Maximum number of affected areas = 8

7.1.2 Vitamin D status and laboratory tests

The serum level of 25(OH)D was adequate (>75 nmol/l) only in 12 patients (23%), while 22 (41%) had vitamin D insufficiency (50-75 nmol/l) and 19 (36%) had deficiency (<50 nmol/l). The frequency of low vitamin D status was the same across the three BMD groups, and there was no difference in the mean vitamin D levels (**Table 7.1**). Also serum calcium and PTH levels were similar in the three BMD groups. Ten patients had elevated PTH levels, and the frequency was higher among osteoporotic patients (p=0.014). Eight of these patients showed abnormal laboratory findings and were subjected to consultation by endocrinologist. Signs of secondary OP were found in five patients and two of them were diagnosed with parathyroid adenoma. A total of five patients had their THA postponed until treated for the calcium imbalance or otherwise cleared for THA.

Patients with low systemic BMD had elevated levels of serum markers for bone formation and resorption, indicating an accelerated bone turnover. Compared to patients with normal systemic BMD, PINP, OCN and NTX levels were significantly elevated (**Figure 7.1**). Increased serum OCN levels were significantly associated with low T-scores at all anatomic sites, except for femoral neck of the OA affected hip (r-values ranging between −0.28 and −0.41, p-values 0.003 - 0.050). High serum levels of resorption markers CTX and NTX were significantly associated with low T-scores in lumbar spine (Spearman correlation, r= −0.33, p=0.017) and in the non-dominant forearm (Spearman correlation, r= −0.31, p=0.034), respectively.

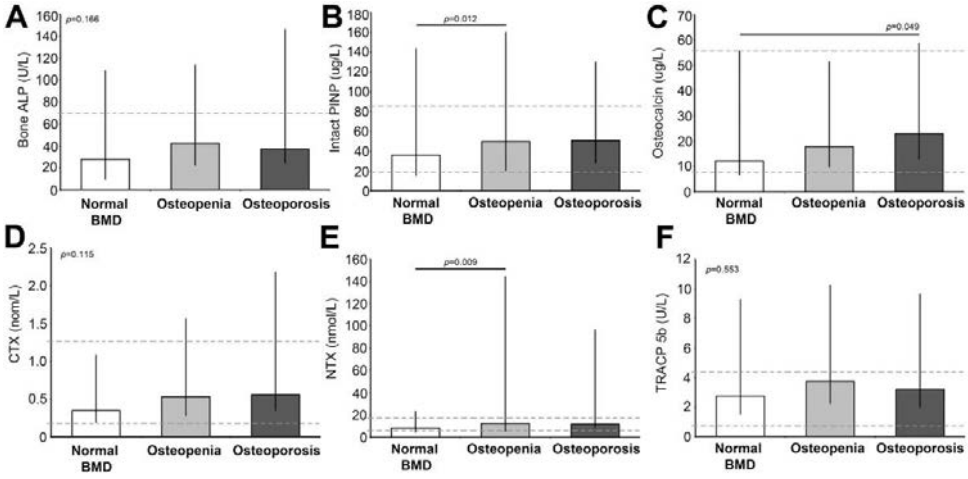

FIGURE 7.1 *Serum levels of bone metabolic markers in patients with normal systemic BMD, osteopenia and osteoporosis. Dashed lines indicate normal range. Kruskal-Wallis test for comparison between groups (general p-value in upper left) and Mann-Whitney U test for pairwise comparisons.*

7.1.3 Characteristics of the OA affected hip and impact of systemic BMD

Radiological classification according to Kellgren-Lawrence score and the disease specific questionnaires Harris hip score and WOMAC showed no difference in the severity, symptoms or degree of functional disability between patients classified with different systemic BMD (**Table 7.3**).

Patients with osteopenia and OP had significantly lower T-scores in their OA affected hips compared to patients with normal systemic BMD (p<0.001)(**Table 7.3**). Analysis of the whole patient population, including those with previous unilateral THA, indicated an increased BMD of the OA affected hip compared with the contralateral side. Paired comparisons in the 39 patients that had underwent bilateral DXA measurements of the hips showed that the femoral necks of the OA affected hips were larger (5.4±0.7 cm^2) and had higher BMC (4.6±0.9 g) compared to the contralateral hips (5.1±0.6 cm^2, p=0.025 and 3.9±0.8g, p<0.001, respectively). On the contrary, the BMC of the trochanteric regions was significantly lower in the OA hips (7.3±1.9g) compared to the contralateral side (7.9±1.6g, p=0.005).

According to the Dorr classification, 23 patients (43%) had normally shaped femurs (Dorr type A) and 25 patients (48%) had femur geometry corresponding to Dorr type B. Five patients (9%) had stove pipe shaped femurs (Dorr type C). The shape of the proximal femur followed the systemic BMD classification (p=0.001), with predominantly type A femurs in the normal BMD group (11 out of 14), high frequency of type B femurs in the osteopenic group (16 out of 24), and type C femurs only found in osteoporotic patients (**Table 7.3**). Patients with Dorr type B and C femurs were significantly older (61±9 years) than patients with Dorr type A femurs (68±7 years, p=0.003).

For the 39 patients included in the prospective part, the CFI was calculated as a quantitative measurement of age-related geometrical changes of the proximal femur. The majority of the patients (N=31) presented with a normal canal flare (CFI 3-4.7)(**Table 7.3**). Eight patients had a stove pipe canal flare (CFI <3), which corresponds to the Dorr type C. These patients were all found in the osteopenic (N=4) and the osteoporotic (N=4) BMD groups. The four patients found to have a champagne flute canal flare (CFI >4.7) were all in the normal BMD group.

TABLE 7.3 *Characteristics of the OA affected hips and disease scores*

	Normal BMD n=14	Osteopenia n=24	Osteoporosis n=15	*p-value* [a]
Kellgren-Lawrence score, n				0.917
2	2	2	1	
3	6	12	7	
4	6	10	7	
Harris hip score, mean±SD	49 (30 – 68)	50 (13 – 84)	49 (24 – 82)	0.982
WOMAC score, mean±SD	47 (35 – 75)	54 (32 – 95)	50 (38 – 73)	0.700
T-scores, mean±SD				
Femoral neck	1.06±0.90	-0.30±0.95	-0.69±1.21	<0.001**
Trochanter	1.13±1.17	-0.07±1.20	-1.21±0.98	<0.001***
Proximal femur	0.77±0.68	-0.30±1.00	-1.20±1.00	<0.001***
Dorr classification, n				0.001**
Type A	11	9	3	
Type B	3	15	7	
Type C	0	0	5	
CFI (N=39), n				0.005**
Champagne flute (>4.7)	4	0	0	
Normal flare (3-4.7)	8	19	4	
Stove pipe (<3)	0	2	2	

[a] One-way ANOVA for continuous variables and chi-square test for categorical variables

**Significant difference between normal BMD and both groups of low BMD

***All pairwise comparisons significant

7.1.4 Microarchitectual and mechanical quality of intertrochanteric cancellous bone (IV)

The quality of intertrochanteric cancellous bone was analyzed by µCT imaging and biomechanical testing of bone biopsies from 35 patients of the primary study population. Structural parameters obtained from µCT data revealed major differences in the microarchitecture of cancellous bone from the implantation site of THA patients (**Table 7.4**). Especially density of the three-dimensional trabecular bone structure was apparent (**Figure 7.2**). These differences were further demonstrated by the wide variation in biomechanical properties (**Table 7.4**).

TABLE 7.4 *Quality of trochanteric cancellous bone evaluated by biomechanical testing and µCT imaging. Mean±SD.*

	Normal BMD n=12	Low BMD n=23 [a]	*p-value* [b]
Biomechanical parameters			
BMD (g/cm³)	1027±35	1023±35	0.734
BV/TV (%)	16±5	13±4	0.057
Tb.Th (mm)	0.18±0.02	0.18±0.02	0.642
Tb.Sp (mm)	0.77±0.14	0.86±0.14	0.089
Tb.N (1/mm)	0.89±0.20	0.73±0.22	0.051
Tb.Pf (1/mm)	5.5±1.1	6.7±1.7	**0.049**
SMI	1.46±0.21	1.66±0.26	**0.026**
DA	2.23±0.36	2.23±0.33	0.938
Microarchitectual parameters			
Failure force (N)	23±19	12±10	**0.033**
Stiffness (N/mm)	41±53	21±21	0.105

[a] Osteopenia (n=18) and osteoporosis (n=5); [b] Student's T test

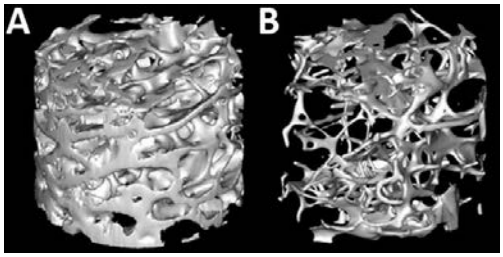

FIGURE 7.2 *Micro-CT reconstruction of intertrochanteric bone biopsies from hip OA patients demonstrating the remarkable difference in three-dimensional trabecular bone architecture of (A) normal and (B) osteoporotic bone.*

Cancellous bone biopsies from patients with low systemic BMD (osteopenic and OP) showed significantly lower ultimate compression failure force and lower stiffness compared to bone biopsies from patients with normal systemic BMD (**Table 7.4**). There were significant differences between the two groups also in microstructural properties, calculated from µCT data. Regression analyses including all 35 patients showed that the microstructural properties of the cancellous bone biopsies, calculated from µCT data, significantly predicted the biomechanical properties (Ultimate compression force and stiffness)(**Table 7.5**).

Biomechanical and microstructural properties of the cancellous bone biopsies also correlated with other basic demographic parameters. Decreased mechanical properties of the cancellous bone biopsies was associated with increased age and low preoperative DXA measured T-score and BMD of the local trochanteric area, explaining 18% (p=0.011), 30% (p=0.001) and 26% (p=0.002), respectively, of the ultimate compression force, and 11% (p=0.049), 25% (p=0.0.004) and 22% (p=0.004), respectively, of the stiffness. There was a weak but significant association between increased age and decreased trabecular thickness (r= −0.36, r^2=0.13, p=0.033). The local DXA measured T-score and BMD of the trochanteric region predicted many of the microstructural µCT parameters. Lower T-score and BMD of the trochanter were associated with decreased bone volume (r^2=0.23, p=0.006 and r^2=0.19, p=0.009), increased trabecular separation (r^2=0.15, p=0.028), decreased trabecular number (r^2=0.28, p=0.002 and r^2=0.22, p=0.005) and increased trabecular pattern factor (r^2=0.15, p=0.030 and r^2=0.14, p=0.025). In contrast, the µCT based BMD of the three-dimensional trabecular bone structure did not correlate with any of the basic demographic parameters. There were no associations between the biomechanical or microstructural properties of intertrochanteric cancellous bone biopsies and the geometry of the proximal femur (Dorr, CFI).

TABLE 7.5 *Relationship between microstructural (micro-CT) and biomechanical properties of cancellous bone biopsies (n=35)*

		Failure force (N)	Stiffness (N/mm)
BMD (g/cm³)	r^2	0.00	0.00
	p	0.932	0.729
BV/TV (%)	r^2	**0.62**	**0.30**
	p	**<0.001**	**0.001**
Tb.Th (mm)	r^2	**0.14**	0.09
	p	**0.029**	0.080
Tb.Sp (mm)	r^2	**0.46**	**0.21**
	p	**<0.001**	**0.006**
Tb.N (1/mm)	r^2	**0.55**	**0.25**
	p	**<0.001**	**0.003**
Tb.Pf (1/mm)	r^2	**0.20**	0.07
	p	**0.007**	0.122
SMI	r^2	**0.27**	0.086
	p	**0.001**	0.087
DA	r^2	**0.14**	**0.13**
	p	**0.030**	**0.033**

r^2= linear regression coefficient of determination; *p*=two-tailed *p*-value

7.2 ISOLATION OF BONE MARROW MSCs AND OPTIMIZATION OF CULTURING PROTOCOLS (II)

7.2.1 Isolation and identification of MSCs

Bone marrow MSCs were isolated from the low-density MNC fraction based on plastic adherence. The first adherent cells were visible after 12-24h. Within 21 days (range 14-21), most cultures reached sub-confluence and cells were detached and re-seeded for further expansion. MSCs were identified according to the minimal criteria. In addition to plastic adherence, MSCs were identified by their colony formation capacity when seeded at low density (200,000 MNCs/cm^2 or 10 passaged MSCs/cm^2) (**Figure 7.3**). By immunocytochemistry, the surface marker profile (CD105+, CD90+, CD73+, CD45-, CD14-) was demonstrated (**Figure 7.3**). The MSC character was further demonstrated by successful trilineage differentiation (**Figure 7.3**).

7.2.2 Higher MSC yield with lower MNC plating density (not previously reported)

Bone marrow samples from 12 patients were used for optimizing the primary isolation culture protocol. By seeding MNCs at a low density (40,000 – 90,000 MNCs/cm^2) a higher MSC yield was obtained from the primary isolation culture (passage 0) compared to cultures seeded at a higher density (120,000 – 190,000 MNCs/cm^2, p=0.013) (**Figure 7.4A**). In the low density cultures the mean (±SD) MSC yield at passage 0 was 128 (±79) MSCs per 10^3 MNCs seeded, whereas the corresponding yield in high density cultures was only 57 (±30) MSCs per 10^3 MNCs seeded. Regression analyses confirmed the inverse relationship between initial seeding density and MSCs yield at the end of passage 0 (r= –0.63, p=0.002), with 40% of the MSC yield explained by the initial seeding density (**Figure 7.4B**). This was further confirmed with regression analysis of all bone marrow samples available (77 cultures from 60 patients; THA patients and reference group, r= –0.36, r^2=0.13, p=0.001).

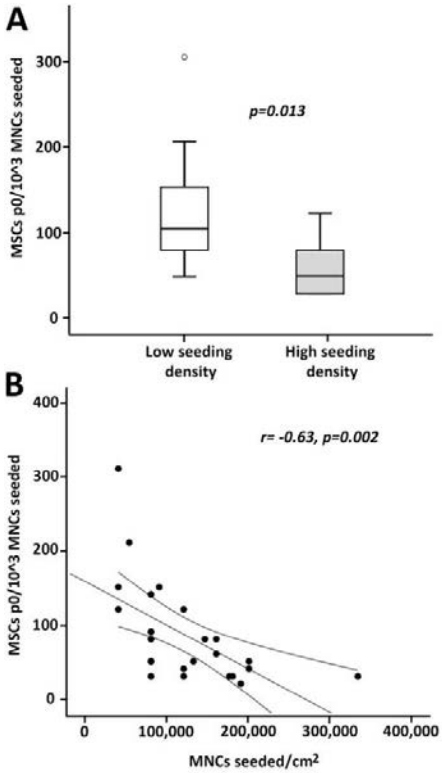

FIGURE 7.4 *The effect of seeding density of MNCs on MSC yield from primary isolation culturing. (A) Higher MSC yield when MNCs seeded at lower (40,000-90,000 MNCs/cm^2) compared to higher (120,000-190,000 MNCs/cm^2) density. Paired T-test, n=12. (B) Correlation analysis demonstrating the significant inverse relationship between MNC seeding density and MSC yield. Pearson correlation, n=25.*

7.2.3 Increased differentiation and decreased variability in osteogenic induction cultures of MSCs by transient 100 nM Dex treatment (II)

7.2.3.1 Timing of Dex supplementation

MSCs from two donors were cultured in osteogenic induction medium (OB medium, containing AA and sodium βGP) supplemented with 10 or 100 nM Dex for different time periods. Differentiation was assessed after 28 days. Strongest differentiation, demonstrated by ALP staining exceeding 80% of the culture areas, was seen in cultures supplemented with 10 or 100 nM Dex for days 1-7, and cultures constantly supplemented with 10 nM Dex (days 1-28)(**Figure 7.5**). In the other conditions only 6-37% of areas were stained for ALP (p<0.001 for all). The degree of mineralization followed a similar pattern, with significantly more mineralization in cultures treated days 1-7 or continuously for 28 days compared to all other treatments (p<0.001 for all). Mean von Kossa stained areas in cultures treated days 1-7 with 10 or 100 nM Dex was 70% and 77%, respectively, followed by 49% and 23% in cultures continuously treated with 10 and 100 nM Dex. With all other treatments, mineralization was negligible (< 5% stained). The only difference between 10 and 100 nM Dex was seen with continuous treatment, where quantified ALP and von Kossa stained areas were over 50% larger with 10 nM compared to 100 nM.

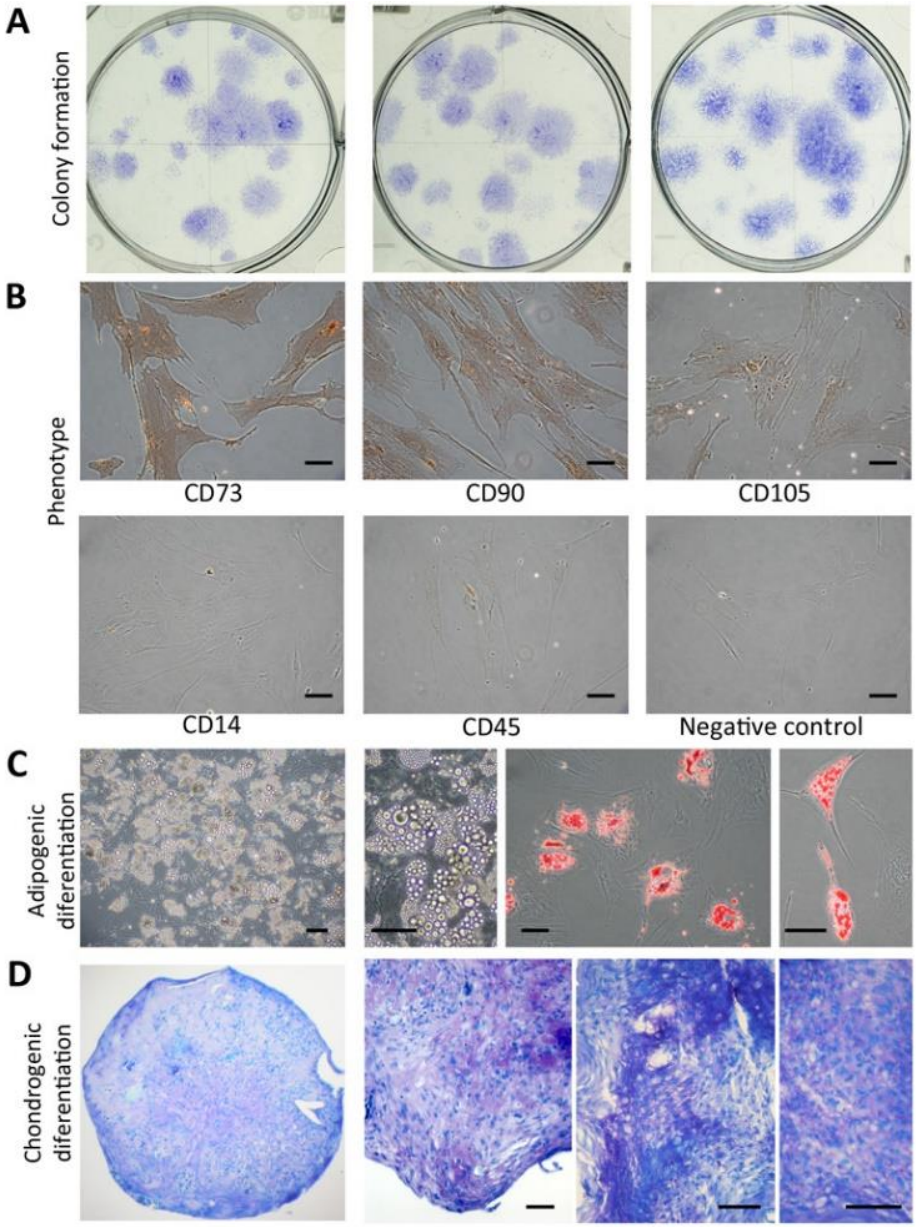

FIGURE 7.3 *MSCs characterization. (A) Colony formation capacity demonstrated by seeding bone marrow MNCs at low density (200,000 MNCs/cm²) in 6-well plates. Representative CFU assays from three THA patients. Crystal violet staining. (B) Immunocytochemistry demonstrating an MSC phenotype according to ISCT minimal criteria. Microphotographs by Terhi Heino. Scale bar=100 µm. (C) Adipogenic differentiation demonstrated upon induction as accumulation of intracellular lipid droplets and staining for Oil Red O. Scale bar=100 µm. (D) Chondrogenic differentiation in micromass cultures, fixed, embedded in paraffin, cut in sections and stained for toluidine blue. Scale bar=50 µm.*

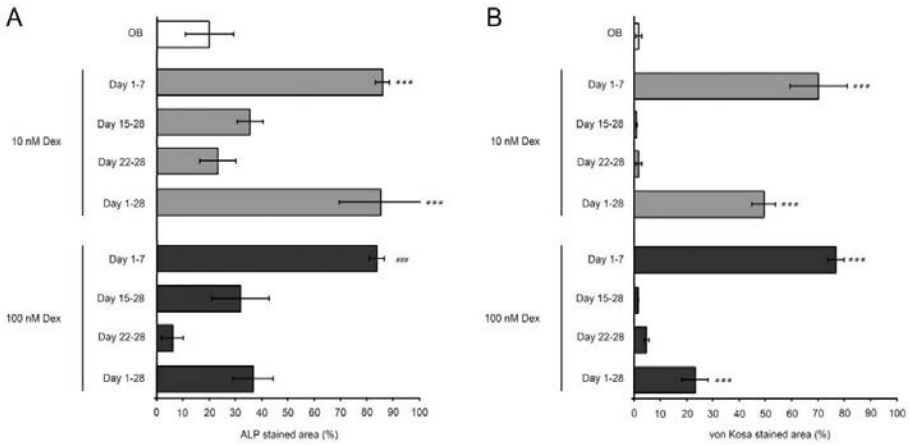

FIGURE 7.5 *Osteogenic differentiation of MSCs after treatment with 10 or 100 nM Dex for varying time periods, compared to control without Dex (OB). Quantification of (A) ALP and (B) von Kossa stained areas. Mean ± SD. Mixed models analyses with Sidak's post hos for pairwise comparisons. ***p<0.001 compared to OB control, 15-28 days and 22-28 days treatments. ### p<0.001 compared to OB control, 15-28 days, 22-28 days and 1-28 days treatments. MSCs from two donors in four replicates per treatment and staining.*

7.2.3.2 Increased ALP activity and mineralization with transient 100 nM Dex

MSCs from 17 donors were cultured in osteogenic differentiation medium supplemented with 10 or 100 nM Dex for the first week (day 1-7) or continuously (day 1-35). ALP activity assay and quantification of ALP and von Kossa stained areas confirmed that transient Dex treatment was more effective compared to continuous Dex treatments. Transient treatment with 100 nM Dex yielded 20-330% higher ALP activity and 8-96% larger ALP stained areas compared to all other osteogenic conditions, whereas transient treatment with 10 nM Dex was less effective with ALP activity 0-70% higher and ALP stained areas of 0-92% higher compared to continuous or no Dex treatments (**Figure 7.6**).

Transient Dex treatment also promoted mineralization. Highest degree of mineralization was obtained with transient 100 nM Dex treatment, showing 41-84% larger von Kossa stained areas compared to other treatments, and 10-14% more staining compared to transient 10 nM Dex treatment. Compared to continuously or no Dex, transient 10 nM treatment showed 31-83% higher von Kossa staining.

Transient treatment with 100 nM Dex yielded the strongest OB differentiation (ALP and mineralization) of MSCs regardless of donor age, as demonstrated by dividing donors into three age groups (**Table 7.6**). Compared to all other conditions investigated, transient 100 nM Dex treatment resulted in highest OB differentiation of MSCs from all donors.

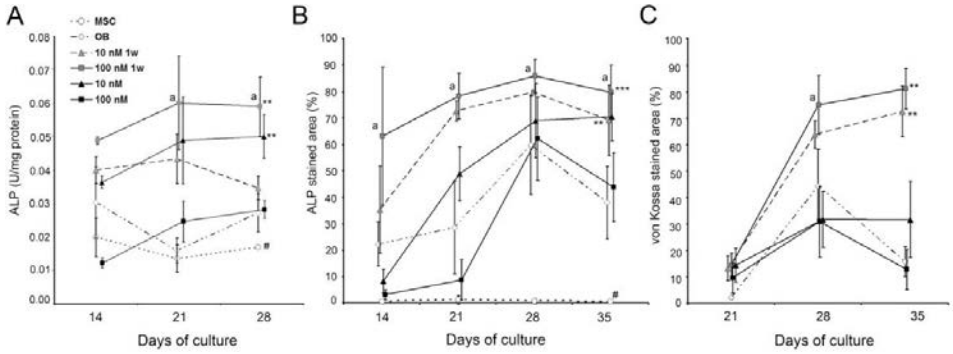

FIGURE 7.6 *Quantification of osteogenic differentiation as function of time. Comparison of transient and continuous treatment with 10 and 100 nM Dex. Osteogenic medium without Dex (OB) and basal medium (MSC) as controls. (A) ALP activity assay, (B) ALP stained area, (C) von Kossa stained area. Mean±SD. Mixed models analysis with Sidak's post hoc for pairwise comparisons. Higher degree of differentiation with one-week Dex treatment (10 and 100 nM) compared to all other treatments. **p<0.01; ***p<0.001; a=100 nM Dex 1w significantly higher compared to 10 nM 1w (p-values from <0.001 to <0.05); #=MSC basal medium lower than all other treatments (p<0.01).*

TABLE 7.6 *Quantification of osteoblastic differentiation in age groups. Mean ± SD*

	Age Group	Days of culture	MSC medium	OB medium	One-week 10 nM Dex treatment	One-week 100 nM Dex treatment	Continuous 10 nM Dex treatment	Continuous 100 nM Dex treatment
ALP stained area (%)	I (19-26 years, n=4)	14	0±0	23±9	50±4	69±8**	11±3	4±2
		21	0±0	18±16	81±4#	83±5***	33±2	16±8
		28	1±1	71±14	88±2	91±2***	75±13	74±6
		35	1±1	47±7	76±7	81±4*	79±11#	66±17
	II (38-60 years, n=6)	14	1±1	23±9	33±9	83±10***	5±3	2±1
		21	2±2	43±17	78±4	85±1*	54±23	8±5
		28	1±1	73±12	81±2	92±5**	83±3	72±5
		35	2±1	42±12	80±3	86±3**	79±6	41±14
	III (65-75 years, n=7)	14	0±0	2±2	23±5#	20±6**	4±2	4±2
		21	0±0	32±1	45±3	52±2**	22±2	1±1
		28	1±1	35±12	61±4	70±5**	18±8	32±4
		35	0±0	23±3	43±4	74±8***	39±15	19±5
von Kossa stained area (%)	I (19-26 yrs, n=4)	21	negative	5±3	15±5	15±4	16±6	15±7
		28	negative	35±18	78±8#	82±8**	42±35	48±31
		35	negative	15±19	82±3	86±2**	16±19	9±5
	II (38-60 yrs, n=6)	21	negative	2±1	15±7#	16±4*	16±7#	13±6#
		28	negative	59±179	61±22#	76±9*	5±3	40±39
		35	negative	24±29	73±6	79±5**	61±19#	14±9
	III (65-75 yrs, n=7)	21	negative	2±1	12±2	11±3	10±5	6±4
		28	negative	16±6	49±21#	64±18*	25±36	6±5
		35	negative	5±3	55±8	73±4**	25±12	4±2

Mixed models analysis of variance, one-week 100 nM Dex treatment compared to all other treatments. Asterisk indicate one-week 100 nM Dex treatment significantly higher compared to all other treatments at that time point; *p<0.05; **p<0.01; ***p<0.001; #Not significantly lower than one-week 100 nM Dex treatment.

7.2.3.3 Transient Dex treatment stimulated proliferation and viability, and inhibited apoptosis

Transient Dex treatment with 10 or 100 nM stimulated cell proliferation compared to continuous Dex treatments. No difference was seen between transient treatment with 10 and 100 nM at any time point. The levels of dead cells were low (< 2%) in all culture conditions throughout the experiment. In MSC medium and cultures continuously treated with 10 and 100 nM Dex the levels of dead cells were elevated at 14 days (still less than 8%). Transient treatment with 100 nM Dex showed the lowest levels of dead cells throughout the experiment. In line with the total fraction of dead cells, apoptosis rates were low in all culture conditions. At day 21, OB-medium without Dex showed significantly higher apoptosis levels compared to all other treatments (5-16 fold). The lowest apoptosis level was found with transient 100 nM Dex treatment.

7.2.3.4 Decreased variability with transient Dex treatment

Compared to other culture conditions, transient treatment with 100 nM Dex resulted in significantly lower variability between donors (interindividual variation) and between parallel samples of the same donor (intraindividual variation). This was evident in all read-out assays of osteoblastic differentiation (ALP-assay, ALP staining, von Kossa staining), independent of donor age (**Table 7.7**).

TABLE 7.7 *Variability within age groups. Inter- and intraindividual coefficients of variation (CV %)* in *osteogenic differentiation assays*

	Age Group	MSC medium	OB medium	One-week 10 nM Dex treatment	One-week 100 nM Dex treatment	Continuous 10 nM Dex treatment	Continuous 100 nM Dex treatment
Interindividual coefficients of variation (%)							
ALP stained area (%)	I	123	49	16	10*	37	54
	II	73	50	27	7*	42	68
	III	183	49	33*	23*	69	69
von Kossa stained area (%)	I	negative	88	8*	8*	110	102
	II	negative	90	43	30*	90	115
	III	negative	65	41*	35*	114	72
Intraindividual coefficients of variation (%), mean (range)							
ALP stained area (%)	I	95 (69-121)	23 (2-44)	7 (1-17)	5 (2-10)*	21 (4-39)	31 (2-60)
	II	75 (68-82)	42 (19-56)	17 (2-38)	6 (0-16)*	19 (2-55)	45 (17-102)
	III	83 (70-96)	23 (2-83)	16 (3-47)§	11 (1-42)§	36 (10-80)	31 (1-50)
von Kossa stained area (%)	I	negative	90 (73-115)	7 (3-12)	2 (1-5)*	93 (53-131)	71 52-82)
	II	negative	49 (4-81)	33 (24-41)	15 (2-39)#	51 (9-92)	60 (21-97)
	III	negative	48 (42-55)	37 (4-80)	19 (6-32)#	46 (6-85)	72 (48-90)

CV% = (SD/mean) x 100; Comparisons between treatments within age groups: *p<0.05 compared to all other treatments; §p<0.05 compared to MSC, 10 nM Dex and 100 nM Dex; #p<0.05 compared to all other but 10 nM 1 week. Age groups: I=19-26 yrs, n=4), II=38-60 yrs, n=6, III=65-75 yrs, n=7

TABLE 7.8 *Qualitative evaluation of osteogenic differentiation in different Dex treatments*

	OB medium	One-week 10 nM Dex treatment	One-week 100 nM Dex treatment	Continuous 10 nM Dex treatment	Continuous 100 nM Dex treatment
Monolayered MSCs	+	+	+	+	+
Cuboidal cells	+	+	+	+	+
Multilayered clusters	+	+	+	+	-
3-D strand-like formations	++	+	+++	+	-
Nodules	-	-	-	-	-
Brownish osteoid	++	++	+	+++	+
Mature mineralized ECM	+	+	+++	+	++
Col1	++	Not done	++	Not done	+
OCN	-	Not done	++	Not done	+

ECM= extracellular matrix; Col1= collagen type 1; OCN=osteocalcin

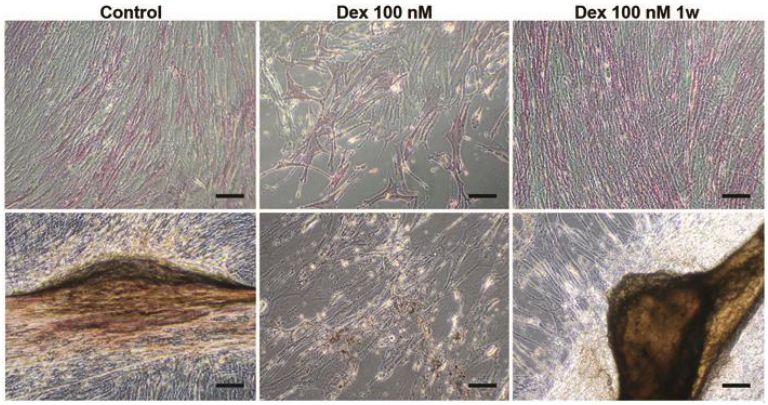

FIGURE 7.7 *Osteogenic differentiation and mineralization in different DEX treated cultures. Clear differences in confluence, cell morphology, and 3D matrix formation were visible between different cultures conditions. Osteogenic differentiation visualized by ALP staining (A-C) and extracellular matrix mineralization visualized by von Kossa staining (D-F). 3D strand-like formations in cultures without Dex (D) and transiently treated with Dex (F) stained strongly for von Kossa. Scale bars=100 µm.*

7.2.3.5 Patterns of in vitro bone formation vary with Dex treatments

Regarding cell morphology and *in vitro* 3D bone matrix formation, clear differences were visible between the culture conditions. Controls without Dex and cultures of one week treatment with 10 or 100 nM Dex reached confluence by day 14, with ALP stained cells evenly spread throughout the culture by day 21. Continuously treated cultures (10 and 100 nM) were only 40% confluent, with mostly single layered cells showing membrane extensions and sparser ALP staining (**Figure 7.7**).

As a further sign of osteogenic differentiation, all cultures but those constantly treated with 100 nM Dex had areas of multilayered cells of both cuboidal and MSC-morphology at day 14. By days 28-35, differences were even more visible regarding the structure and morphology of 3D formations associated with bone formation. Typical bone nodules were rarely observed. In cultures transiently treated with 100 nM Dex multilayered cells packed tightly to form 3D strand-like formations that stained positive for both ALP and von Kossa. Similar formations were observed in control cultures without Dex (**Figure 7.8**). Cultures constantly treated with 100 nM Dex showed no 3D matrix formation.

Immunostaining for COL1 and OCN confirmed complete osteoblastic differentiation. At day 14, COL1 staining was clear and widespread in controls without Dex and cultures transiently treated with 100 nM Dex. As expected, OCN was not detected in cultures without Dex at any time point. Cultures transiently or continuously treated with 100 nM Dex stained positive for OCN at 28 days. A summary of quantitative evaluation with different Dex treatments are presented in **Table 7.8**.

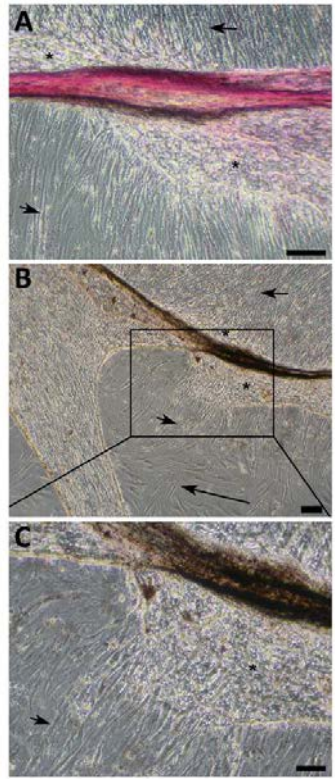

FIGURE 7.8 *Three dimensional strand-like formations were seen in all cultures transiently treated with Dex. These 3D structures, representing the sites of in vitro bone formation, stained strongly for both ALP (A) and von Kossa (B-C). The strand-like formations were surrounded by multilayer of cells (*) together with a few round cells. Further out from the 3D structures were undifferentiated monolayer of cells with MSC morphology (arrows). The rectangle area in (B) is magnified in (C). Scale bars=100 µm.*

7.3 BONE MARROW MSCs IN THA PATIENTS (VI)

7.3.1 Sample collection and progress through study protocol (not previously reported)

Bone marrow aspirate was obtained from 49 of the 53 eligible patients. Unsuccessful bone marrow sampling (n=4) was associated with obesity which technically compromised aspiration. From the bone marrow sample of eight patients no growing cells were obtained. Five of these samples were associated with difficulties in aspiration. Common for these eight samples was a low yield of MNCs after density centrifugation (<10 million MNCs), providing a poor starting material for MSC isolation. MSCs were successfully isolated and culture expanded through several passages from bone marrow samples of 41 patients to obtain growth data in terms of population doublings. Due to the high numbers of cells needed for each assay, samples from all patients could not be included in every experiment. Osteoblastic differentiation data was obtained from 30 patients.

7.3.2 Large variability in *in vitro* properties of MSCs from postmenopausal THA women

Iliac crest bone marrow MSCs from postmenopausal females undergoing cementless THA exhibited large individual variability in cell yield, growth kinetics, and osteogenic differentiation, with coefficients of variation ranging between 31% and 172% for the different parameters. Properties of MSCs from this group of THA patients were significantly decreased, approximately 50%, compared to MSCs from the reference group of younger premenopausal females (mean age 40±16, range 19-60) from our previous studies (Alm et al., 2010, Laine et al., 2012).

7.3.2.1 Cell yield and proliferative capacity (not previously reported)

Although the volume of bone marrow aspirated from THA patients was larger compared to the reference group (p=0.001), the yield of MNCs after density centrifugation was significantly lower (p=0.007), despite broad variations within the THA group. The mean yield of MSCs from the primary isolation culture (passage 0) was 168 cells per 10^3 MNCs seeded, ranging from 10 to 1,325 cells, which was not different from the reference group (p=0.108)(**Table 7.9**). Based on the CFU assay (2x10^5 MNCs/10 cm^2), anticipating that each colony is derived from one MSC, the number of MSCs in the bone marrow represented on average 0.005% (±0.003%) of the MNCs, ranging from 0.001 to 0.012% for the individual THA patients. This was similar to that of the reference group (p=0.124).

The proliferative capacity of MSCs from THA patients, assessed as PDs at each passage, varied widely (**Figure 7.9**). MSCs from individual THA patients displayed different patterns of cumulative growth kinetics, demonstrating generally low expansion capacity. The maximum number of PDs reached during a single passage (p1-3) was on average 3.7 (range 1-6.9), coupled with a slow growth rate (**Table 7.9**).

The CFU assay was performed repeatedly at several passages (P0-3) for monitoring the preservation of the capacity to form colonies when seeded at low density through culture expansion. In line with observation for other properties, MSCs from the different THA patients exhibited varying colony formation capacity, with a decreasing trend following increased expansion. By passage 3, the CFU capacity had decreased significantly compared to passage 0 (p=0.049) (**Table 7.10**).

TABLE 7.9 *Cell yield and MSC growth characteristics, mean (range)*

	THA females n=41	Reference group (younger healthy female donors, n=8)
Bone marrow aspirate (ml)	4.8** (3 – 9)	3.5 (2 – 4.5)
MNCs/ml bone marrow	2.8 x 10^6** (0.2 – 30 x 10^6)	17.3 x 10^6 (4.2 – 34 x 10^6)
MSCs p0/10^3 MNC plated [a]	168 [NS] (14 – 1325)	96 (30 – 280)
CFU/BM MNCs (%)	0.005 ± 0.003 [NS]	0.001±0.012
Max PDs	3.7 [NS] (1.0 – 6.9)	3.6 (2.3 – 4.7)
Max PD-rate (PDs/day)	0.13 (0.02 – 0.27)**	2.24 (0.07 – 0.41)
Age (mean±SD, range)	65±9 (41-79)**	37±17 (19-60)

Comparisons performed using Mann-Whitney U test; Asterisks indicate comparison to the reference group *p<0.05, **p< 0.01, ***p< 0.001, NS = Not statistically significant.

TABLE 7.10 *Colony formation capacity through passaging*

Passage	Mean	Range
P0	8.0	2.7-17
P1	6.7	1.7-20
P2	6.1	0-16
P3	1.2*	0-2.7

Colony formation capacity= number of CFUs/100 MSCs seeded at 10 cm^2, mean of triplicates; *p=0.049 compared to P0, Mann-Whitney U test

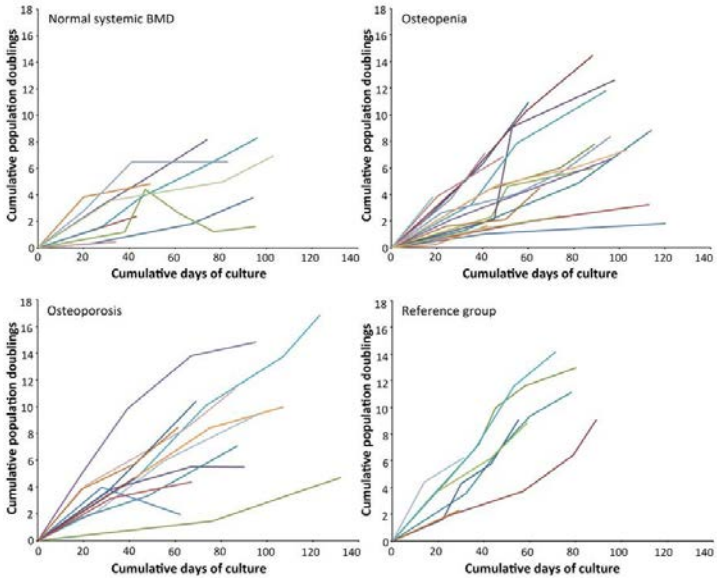

FIGURE 7.9 *The proliferative capacity of MSCs expressed as cumulative population doublings. The proliferative capacity varied widely for MSCs from individual THA patients with normal systemic BMD (n=9), osteopenia (n=19), and osteoporosis (n=13), with a generally lower capacity compared to MSCs from the reference group of younger female fracture patients (n=8).*

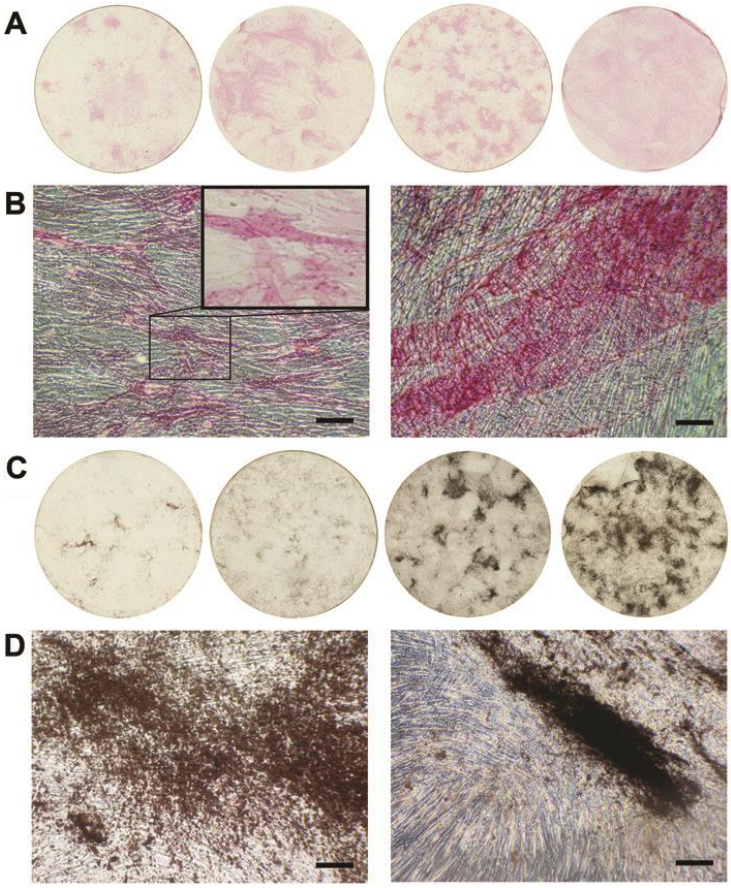

FIGURE 7.10 *Osteogenic differentiation of MSCs from THA females. After culturing under osteogenic conditions, differentiation was visualized by staining for ALP (A-B). Indicating osteogenic maturation, mineralized extracellular matrix was visualized by staining for von Kossa (C-D) MSCs from individual patients cultured in 24-well plates (Ø 15.5 mm) showed varying degree of (B) differentiation and (C) mineralization, which was utilized for dividing the patients into high and low OB-capacity groups. Scale bars=100 µm.*

7.3.3 Osteogenic differentiation capacity

Osteogenic differentiation was demonstrated by staining for ALP (**Figure 7.10A-B**), and by spectrophotometric measurement of ALP-activity. As a measure of complete differentiation into functional OBs, extracellular matrix mineralization was analyzed with von Kossa staining (**Figure 7.10C-D**) and spectrophotometric quantification of deposited calcium.

MSCs from individual THA patients showed major variability in their osteogenic capacity. Cells from all donors demonstrated osteogenic differentiation, although at varying degree. The ALP stained area quantified after 4-5 weeks of induction was on average 39±26%, ranging from 1 to 90% (n=30) (**Table 7.11**).

The level of mineralization was generally low and complete mineralization was detectable only for MSCs from part of the patients, with a mean von Kossa stained area of 31% (range 5-55%) (**Table 7.11**). From 13 patients, no matrix mineralization could be detected despite repeated osteogenic culturing at two or three different passages (P1-3). These MSCs were hence classified as negative for mineralization.

TABLE 7.11 ***In vitro osteogenic capacity of iliac crest BM-MSCs from THA patients (n=30)***

	Mean ± SD	Range
ALP activity (U/g protein)[#]	11 ± 6	6-22
ALP stained area (%)	39 ± 26	1-90
von Kossa stained area (%)	11 ± 17[a] 31 ± 16[b]	0-55 5-55
Ca (mmol/l)[&]	0.19 ± 0.18	0.06-0.64

[#] n=25; [&] n=23; [a]All donors, including zero values; [b]Only values >0 included (N=17); Reference group (mean, range): ALP activity= 99 U/g (47-250), ALP staining= 84% (57-90), von Kossa=77% (41-89), Ca=2.3 mmol/L (0.24-3.4).

7.3.4 In vitro osteogenic differentiation capacity of MSCs correlate with clinical bone quality parameters

Within the THA patient group, there were no significant age-related effects on any MSC parameter, despite an age range between 41 and 79 years. To further investigate the relevance of experimental MSC capacity in relation to bone health of the donors, ALP stained areas as a measure of *in vitro* osteogenic differentiation capacity was analyzed against demographic and clinical bone quality parameters using linear correlation. MSCs from subjects with low preoperative T-score showed decreased *in vitro* osteogenic differentiation (r=0.42, p=0.019, n=30). There was an inverse relationship between *in vitro* ALP expression and serum levels of vitamin D (25(OH)D)(r= −0.42, p=0.021, n=30). Further, ALP levels in MSC cultures correlated with serum levels of bone formation marker ALP (r=0.38, p=0.045, n=27) and bone resorption marker TRACP-5b (r=0.50, p=0.006, n=27).

Patients whose MSCs failed to produce mineralized matrix *in vitro* (n=13) had significantly lower systemic BMD (lowest T-score −3.1±1.6) compared to patients with MSCs capable of *in vitro* mineralization (n=17)(lowest T-score −1.9±1.1, p=0.044). There were no differences in serum levels of bone turnover markers, 25(OH)D or PTH levels between the mineralization groups. There were no associations between *in vitro* osteogenic differentiation (ALP or mineralization) with age, BMI or PTH in the present patient population.

7.4 TWO-YEAR CLINICAL FOLLOW-UP OF THA PATIENTS (III-V)

Of the 53 patients enrolled in the initial screening process, 43 patients fulfilled the criteria for the prospective part of the study, and ten patients with previously undiagnosed severe OP were added to the off-trial subgroup. Of the 43 patients, three had revision surgery due to periprosthetic fractures. Of these patients, one had normal systemic BMD and two had low systemic BMD. Two patients were excluded from the DXA-measurement of periprosthetic BMD (***Study III***) due to omitted baseline measurement, and one patient could not complete the RSA follow-up (***Studies IV-VI***) due to absence of RSA bone markers.

7.4.1 Clinical outcome

Subjective evaluation of functional outcome of the THA (N=40), assessed by disease scores, showed significant improvement in both Harris hip score and WOMAC score (**Table 7.12**) already at 3 months ($p<0.001$ for both), with continued improvement up to 24 months ($p<0.001$ for both). The mean HHS improved from 50 (13-84) preoperatively to 70 (21-100) at 3 months and 85 (42-100) at 24 months. The mean WOMAC score improved from 50 (16-95) preoperatively to 24 (4-77) at 3 months and 16 (0-59) at 24 months. There were no differences in functional outcome scores between the three BMD groups. Eight patients reported moderate pain at 24 months, and one reported extreme. According to the total score at 24 months, two patients with low systemic BMD reported moderate residual symptoms, while all other patients (95%) reported minimal or mild symptoms.

TABLE 7.12 ***Subjective evaluation of functional outcome of THA assessed by disease scores in patients with normal or low preoperative systemic BMD, mean (range)***

	Normal BMD (n=12)	Low BMD (n=27)	*p*-value [a]
HARRIS HIP SCORE (higher score indicate milder disease)			
Preoperatively	49 (30-68)	51 (13-84)	0.844
3 months	75 (33-98)	75 (21-100)	0.976
6 months	87 (63-100)	85 (59-100)	0.659
12 months	87 (74-100)	88 (50-100)	0.876
24 months	86 (42-100)	85 (53-100)	0.881
WOMAC score (low score indicate milder disease)			
Pain sub scores (0 – 20)			
Preoperatively	10 (6-18)	11 (6-20)	0.339
3 months	6 (0-20)	5 (0-13)	0.659
6 months	3 (0-8)	4 (0-13)	0.630
12 months	2 (0-6)	3 (0-12)	0.888
24 months	2 (0-6)	4 (0-17)	0.098
Stiffness sub score (0 – 8)			
Preoperatively	4 (2 – 7)	5 (3 – 8)	0.096
3 months	3 (0 – 6)	3 (1 – 6)	0.737
6 months	2 (0 – 4)	2 (0 – 6)	1.000
12 months	2 (0 – 4)	2 (0 – 5)	0.798
24 months	1 (0 – 3)	2 (0 – 5)	0.086
Physical function sub score (0 – 68)			
Preoperatively	33 (27 – 50)	37 (16 – 37)	0.273
3 months	19 (3 – 51)	17 (6 – 54)	0.697
6 months	14 (0 – 27)	14 (3 – 46)	0.884
12 months	8 (0 – 14)	11 (0 – 30)	0.096
24 months	9 (0 – 21)	12 (0 – 37)	0.253
Total scores (0 – 96)			
Preoperatively	47 (35-75)	53 (32-95)	0.322
3 months	26 (5-77)	25 (7-73)	0.591
6 months	19 (0-38)	20 (4-61)	0.499
12 months	10 (0-21)	16 (0-44)	0.662
24 months	13 (2-30)	17 (0-59)	0.214

[a] Student's T-test

7.4.2 Periprosthetic BMD

During the first 6 months after surgery, there was a general bone loss around the femoral stem, followed by recovery in most zones by 24 months (**Figure 7.11**). Total periprosthetic BMD (zones 1-7 together) decreased by 3.8% by 6 months (p=0.003), but started to recover thereafter, reaching -2.6% at 12 months (p=0.002) and approached the baseline value by 24 months (-1.5%).

The most prominent changes in periprosthetic BMD was seen in zones 5 and 7. In zone 5, BMD increased continuously compared to baseline, with a bone gain of 2.2% at 6 months (p=0.007) and 5.2% at 24 months (p < 0.001). In Gruen zone 7, a continuous bone loss was seen with an average BMD decrease of 16% already at 3 months (p<0.001). At 24 months, BMD in zone 7 had decreased on average by 23% (p < 0.001). On radiographs, bone loss in zone 7 was detected as cortical bone rounding, a sign of adaptive remodeling following stem implantation (**Figure 7.12)**. Low preoperative systemic BMD predicted this bone loss in zone 7 (p=0.037) (**Figure 7.11**).

Regression analysis between the lowest preoperative T-score (any measured site) and BMD changes in zone 7 at 24 months confirmed that low preoperative systemic BMD was associated with higher bone loss (r=0.38, r2=0.147, p=0.015). The preoperative total BMD of the operated hip did not act as an independent predictor of periprosthetic bone loss in zone 7 (p=0.648) and no association was found between baseline BMD and loss of BMD for zone 7 at 24 months (p=0.994).

Preoperative serum markers of bone turnover failed to predict bone loss in zone 7. On the contrary, there was a relationship between bone turnover markers and the transient BMD decrease in zone 2 as well as the continuous BMD increase in zone 5. In zone 2, high preoperative levels of resorption markers NTX and TRACP-5b (p<0.001 for both), and low preoperative level of bone formation marker PINP (p=0.01) predicted the transient bone loss during the first 6 months. Also the uncoupling index predicted the BMD changes in zone 2 (p=0.002), with better preservation of periprosthetic bone in patients with turnover in favor of bone formation (positive index) compared to patients with turnover in favor of bone resorption (negative index). In zone 5, high preoperative serum levels of bone ALP predicted the continuous increase of BMD (p=0.04).

No associations were found between any of the radiographic parameters of the proximal femur geometry (Dorr classification and CFI) and degree of periprosthetic bone loss.

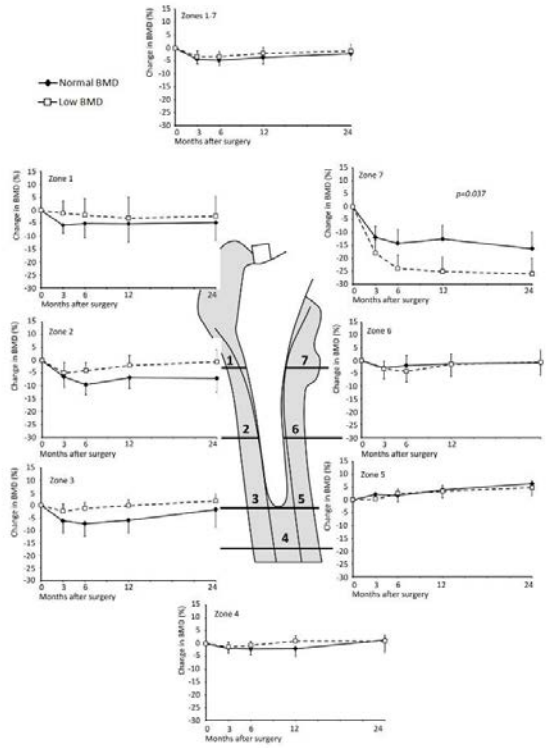

FIGURE 7.11 *Mean (95% CI) percentage change in periprosthetic BMD in each of the seven Gruen zones and in total periprosthetic BMD (1-7) in patients with normal (solid line) or low (dashed line) systemic BMD. Low systemic BMD predicted increased bone loss in Gruen zone 7.*

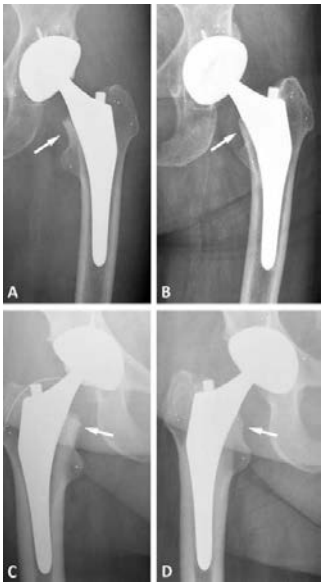

FIGURE 7.12 *Adoptive remodeling. Radiographs taken postoperatively (A, C) and after 2-years (B, D) demonstrating bone changes in Gruen zone 7 in two of the THA patients. Bone loss is seen as rounding off of the medial periprosthetic bone (arrows) due to adaptive remodeling.*

7.4.3 Magnitude of stem migration compared to baseline

A total of 39 patients completed the 24 months RSA follow-up. Stem migration mainly took place during the first 3 months after surgery, and in 35 patients the stem had migrated beyond the detection limits for micromigration (translation and/or rotation). The mean (±SD) initial stem subsidence of all patients was 0.9±0.8 mm, ranging between 0 and 3.9 mm ($p<0.001$ compared to baseline). The mean axial rotation at 3 months was 0.8±2.0 degrees, ranging between 0.1 and 8.5 degrees ($p=0.03$ compared to baseline). Of the stems that rotated beyond the detection limit, rotation was retroversion in 17 patients and anteversion in four patients.

When comparing the stem positions at 24 months to baseline, there appeared to be no significant additional migration after 3 months, as the average change in stem position along the y-axis (y translation) at 24 months was 1.0±0.9 mm (range 0.0-4.2 mm), and the average change in stem position around the y-axis (y rotation) was 1.8±1.8 degrees (range 0.1-8.9 degrees). However, analyses at the individual patient level indicated otherwise. At 3 months, subsidence exceeding 2 mm was detected in five of the patients, and by 24 months two additional patients displayed subsidence of more than 2 mm. This was even more pronounced for the axial rotation, where only two patients had rotational migration exceeding 3 degrees at 3 months, but by 24 months the stems in a total of 12 patients had rotated more than 3 degrees.

7.4.4 Effect of systemic BMD on stem migration

Preoperative systemic BMD status predicted the magnitude of stem subsidence ($p=0.007$), with more initial stem subsidence in patients with low systemic BMD compared to patients with normal systemic BMD (**Figure 7.13A**). The difference between the BMD groups remained during the 24-months follow-up. The three dimensional translational migration (x, y, z), represented by the translation vector, showed a similar trend of more migration in patients with low systemic BMD, but the difference was not statistically significant ($p=0.1$) (**Figure 7.13B**). In both BMD

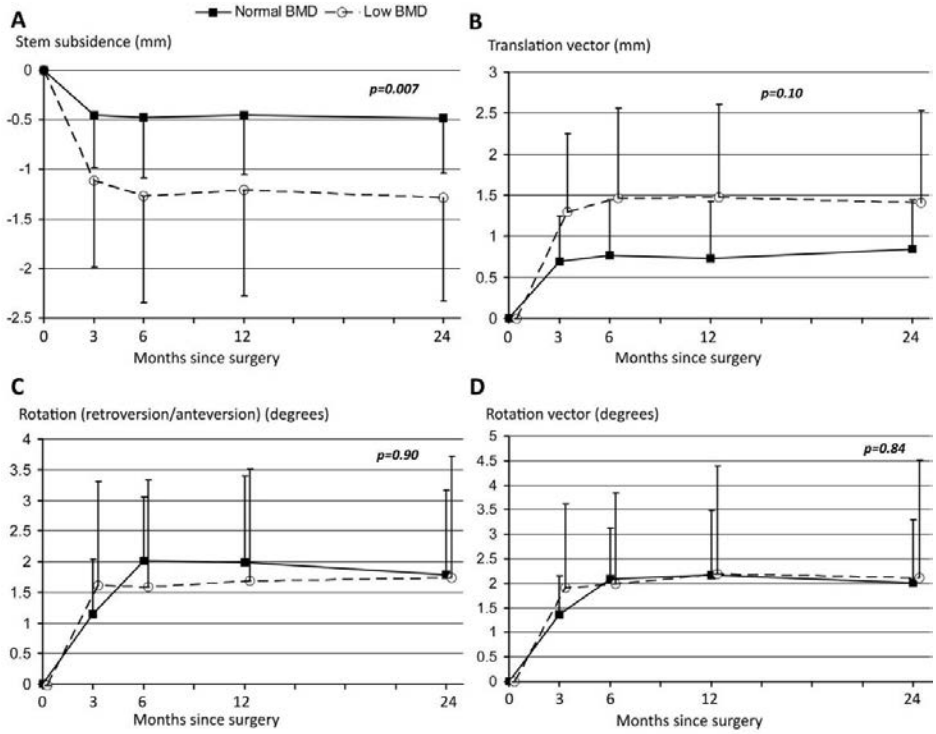

FIGURE 7.13 *Time related stem migration determined by RSA in THA patients with normal (solid line) or low (dashed line) systemic BMD. (A) Axial migration expressed as stem subsidence (precision 0.42 mm). (B) Total translational migration expressed as the translation vector (precision 0.40 mm). (C) Axial rotation of the stem (retroversion or anteversion, precision 1.81 degrees). (D) Total rotational migration expressed as the rotation vector (precision 1.32 degrees). Time related differences between groups analyzed with repeated measures ANOVA with group as cofactor. Time related changes from baseline to 3 months analyzed with paired T-test. Time related changes between 3 and 24 months analyzed with repeated measures ANOVA. Mean±SD.*

groups, translational migration was significant at 3 months compared to baseline (p<0.001 for both), with additional moderate migration from 3 to 24 months (p=0.03 for both).

Axial rotation of the stems was significant at 3 months both in patients with normal and low systemic BMD (**Figure 7.13C**). Preoperative systemic BMD did not predict the magnitude of rotation (p=0.9). In the normal systemic BMD group, stems continued to rotate up to 6 months (p=0.001 compared to 3 months), but not thereafter.

Corresponding to the migratory pattern in the other RSA parameters, also three dimensional rotational migration (x, y, z), assessed as the rotation vector, was significant at 3 months compared to baseline in both BMD groups (p<0.001 for both) (**Figure 7.13D**). In patients with normal systemic BMD additional rotation took place between 3 and 6 months (p<0.001) with additional minor further rotation up to 24 months (p=0.030). In the low systemic BMD group no further migration was observed after the 3 months settling period. Despite that, there was no difference in magnitude of total rotational migration between patients with normal and low systemic BMD (p=0.8).

7.4.5 Quality of intertrochanteric cancellous bone as predictor of stem migration

Surprisingly, the quality of the local intertrochanteric bone, assessed by µCT and biomechanical analyses of bone biopsies, did not predict the magnitude of translational or rotational migration. Only when µCT-based BMD was analyzed as quartiles (low, middle, high BMD), it became evident that higher µCT-based BMD was weakly associated with increased stem subsidence (r=0.40, r^2=0.16, p=0.022), and increased total translational migration (translation vector) (r=0.36, r^2=0.13, p=0.038). Logistic regression further identified high µCT-based BMD as a risk factor for increased stem subsidence (OR 0.1, 95%CI 0.01 to 0.064, p=0.019) and axial rotation (OR 10.4, 95%CI 1.4 to 76.1, p=0.021).

7.4.6 Stem osseointegration

Assessment of stem osseointegration at the individual patient level was performed by classifying each patients' stem as fixed (osseointegrated) or migrating by comparing the stem position to the position at the presiding time point. Osseointegration was primarily evaluated as translational and rotational stability separately based on the corresponding vectors, representing 3D migration. In 7 out of 39 patients translational stability (translation vector ≤ 0.40 mm) was detected at 3 months, representing the time course of normal osseointegration. In the majority of patients (N=24) the stem had stabilized by 6 months, corresponding to delayed osseointegration. In eight patients, stems continued to migrate beyond 6 months. Four of these stabilized by 12 months (late osseointegration), and in four patients migration was still detectable at 24 months, indicating instability.

Rotational stability (rotation vector ≤ 1.32 degrees) was detected in 17 patients at 3 months, and three patients achieved rotational stability by 6 months (delayed osseointegration). Of the remaining 19 patients, eight had late rotational stability. In two patients, migration was still detectable at 24 months.

When combing all four RSA parameters (y translation, y rotation, translation vector, and rotation vector), stability by 3 months (normal osseointegration) was achieved in 20 patients. In 19 patients stems continued to migrate beyond 6 months. Four of these stabilized by 12 months, and eight stabilized by 24 months (**Table 7.13**). In seven patients, stems had not stabilized by the 24 months end-point; two patients with normal systemic BMD and five with low systemic BMD. On conventional radiographs, none of these patients showed classical signs of stem loosening. All of these patients reported slight or moderate pain.

Kaplan-Meier time-to-event-estimates showed that the probability of translational (vector) stability within 3 months was significantly higher in patients with normal systemic BMD (50%) compared to patients with low systemic BMD (4%, p=0.015). The probability of rotational (vector) stability within 3 months was similar in the two groups, with a 42% probability for patients with normal systemic BMD and a 44% probability for patients with low systemic BMD. The local µCT-based BMD quartiles did not affect the probability of translational or rotational stability.

Based on logistic regression analyses, low systemic BMD (OR=0.1, CI 0.006-1.0, p=0.02), aging (OR=1.1, CI 1.0-1.2, p=0.02) and low local hip BMD adjusted for age (OR=0.3, CI 0.1-0.7, p=0.005) were identified as risk factors for delayed translational stability, while aging (OR=3.0, CI 1.1-9, p=0.04) and low CFI (stovepipe geometry) (OR=1.1, CI 1.0-1.2, p=0.02) were risk factors for delayed rotational stability.

TABLE 7.13 *Stem osseointegration. Number of stable stems at different follow-up time points (n=39)*

Time point	Number of stabile stems	Classification of osseointegration
3 months	4 (10%)	Normal osseointegration
6 months	16 (41%)	Delayed osseointegration
12 months	4 (10%)	Late osseointegration
24 months	8 (21%)	
Not stable at 24 months	7 (18%)	Unstable stems

7.5 OSTEOGENIC CAPACITY OF MSCs AND OSSEOINTEGRATION OF CEMENTLESS FEMORAL STEMS (VI)

Of the 30 patients with successful MSC analysis, eleven were excluded from the RSA follow-up due to; initiation of antiresorptive drug therapy for severe OP (N=9), surgical complication (N=1), or absence of RSA markers (N=1). Hence, the number of patients with concomitant successful MSC analysis and completed RSA follow-up was 19 (age 65±8, 50-78 years), including four with normal systemic BMD, 12 with osteopenia and three with OP. All patients had femur morphology corresponding to Dorr type A (N=12) or B (N=7). For the purposes of analyzing the relationship between *in vitro* osteogenic differentiation capacity of MSCs and hip implant migration, patients were classified as having MSCs with high (N=7) or low (N=12) osteogenic (OB) capacity (**Table 7.14**).

TABLE 7.14 *Demographic and clinical characteristics of THA patients with low and high in vitro osteogenic capacity of their bone marrow MSCs (n=19) (Study VI)*

Demographics (Mean ± SD, range)	**Low OB-capacity MSCs (n=12)**	**High OB-capacity MSCs (n=7)**	***p*-value[a]**
Age	66 ± 8 (50-78)	63 ± 7 (55-73)	0.520
BMI	31 ± 5 (23-39)	30 ± 6 (24-38)	0.708
S-25(OH)D (nmol/l)(normal, 50-140)	52 ± 19 (30-93)	65 ± 33 (19-106)	0.294
S-PTH (ng/l) (normal, 10-55)	40 ± 12 (20-58)	37 ± 7 (24-45)	0.682
Lowest T-score	−1.7 ± 1.0 (−3.5 to −0.2)	−1.4 ± 0.9 (−2.4 to 0.2)	0.531
Bone mineral density (g/cm²)			
Contralateral hip, femoral neck	0.78 ± 15(0.62-1.11)	0.79 ± 0.09 (0.71-0.95)	0.919
Non-dominant forearm, total distal radius	0.54 ± 0.05 (0.43-0.62)	0.54 ± 0.06 (0.49-0.62)	0.849
Lumbar spine, total	1.10 ± 0.17 (0.71-1.44)	1.03 ± 0.14 (0.87-1.18)	0.788
Serum markers of bone turnover (Mean ± SD, range)			
Bone ALP (U/l) (normal <69)	37 ± 19 (13-81)	36 ± 14 (21-57)	0.911
Intact PINP (µg/l) (normal, 19-84)	51 ± 22 (26-97)	59 ±21 (45-98)	0.501
OCN (µg/l) (normal, 8-56)	23 ± 12 (6-43)	20 ± 7 (14-30)	0.590
CTX (nM/l) (normal, 0.112-0.738)	0.54 ± 0.32 (0.20-1.29)	0.46 ± 0.12 (0.35-0.65)	0.553
NTX (nM/l) (normal, 6.2-19)	11 ± 4 (4-18)	10 ±4 (7-16)	0.920
TRACP-5b (U/l) (normal, 1.4-4.2)	3.7 ± 1.4 (1.2-5.6)	4.1 ±1.3 (2.8-6.5)	0.533
Frequencies, n (%)			***p*-value[b]**
Systemic BMD			0.308
Normal BMD	3 (25%)	1 (14%)	
Osteopenia	6 (50%)	6 (86%)	
Osteoporosis	3 (25%)	0 (0%)	
Dorr classification of proximal femur geometry			0.960
Type A	7 (58%)	4 (57%)	
Type B	5 (42%)	3 (43%)	
25(OH)D insufficiency (<50 nmol/l)	6 (50%)	3 (43%)	0.502
Previous fracture	4 (33%)	1 (14%)	0.394
Estrogen replacement	4 (33%)	1 (14%)	0.457
Smoking	1 (8%)	1 (14%)	0.467
Alcohol consumption, > 3 drinks/week	2 (17%)	2 (28%)	0.828
Disease-specific scores (Mean ± SD, range)			***p*-value[a]**
Harris hip score			
Preoperative	53 ± 18 (17-84)	52 ± 15 (26-68)	0.902
24 months postoperatively	79 ± 21 (42-100)	89 ± 10 (74-100)	0.289
WOMAC score			
Preoperative	51 ± 13 (32-77)	62 ± 18 (39-77)	0.213
24 months postoperatively	21 ± 19 (0-59)	16 ± 10 (6-26)	0.613

[a] *p* = two-tailed p-value from Student's T-test; [b] p-value from chi-square test; OB = osteogenic differentiation; OB-capacity groups based on combined quartiles from ALP and mineralization; ALP= alkaline phosphatase; PINP=procollagen type I propeptide; OCN= Osteocalcin; CTX=C-terminal crosslinking telopeptide of type I collagen; NTX=N-terminal crosslinking telopeptide of type I collagen; TRACP-5b= Tartrate-resistant acid phosphatase type 5b

TABLE 7.15 *RSA-measured femoral stem migration (change in stem position) in patients with low and high OB-capacity of their bone marrow MSCs, mean ±SD (Study VI)*

	Low OB-capacity (n=12)	High OB-capacity (n=7)	Mean difference	95% confidence interval	*p*
Stem subsidence (y)(mm) (detection limit 0.42 mm)					
3 months	1.4 ± 1.2*	0.6 ± 0.5	0.8	–0.1 to 1.6	0.070
24 months	1.5 ± 1.2*	0.6 ± 0.7	0.9	–0.1 to 1.9	0.083
Translation vector (x,y,z) (mm) (detection limit 0.40 mm)					
3 months	1.6 ± 1.3**	0.7 ± 0.6	0.9	–0.05 to 2.0	0.060
24 months	1.8 ± 1.2**	0.8 ± 0.6	0.9	–0.04 to 1.9	0.058
Stem rotation (y)(degrees) (detection limit 1.81 degrees)					
3 months	1.5 ± 0.8	1.1 ± 1.2	0.4	–0.5 to 1.4	0.366
24 months	2.1 ± 1.7	0.9 ± 0.6	1.1	–0.2 to 2.4	0.058
Rotation vector (x, y, z) (degrees) (detection limit 1.32 degrees)					
3 months	1.8 ± 0.8	1.2 ± 1.1	0.6	–0.3 to 1.5	0.193
24 months	2.6 ± 2.2	1.2 ± 0.5	1.4	–0.2 to 3.0	0.074

Change in stem position along the y-axis (proximal/distal) represents stem subsidence; Change in stem position around the y-axis (retroversion/anteversion) represents stem rotation; Vectors represent three-dimensional migration along (translation) or around (rotation) three axes (x, y, z); Stem subsidence and rotation are presented as mean of absolute (unsigned) values; Asterisks indicate migration significantly exceeding the detection limit, i.e. the stability limit, compared to baseline (*p<0.05, **p<0.01); OB = osteogenic differentiation; OB-capacity groups based on combined quartiles from ALP and mineralization; *p* = two-tailed p-value from Student's T-test for comparison between the low and the high OB-capacity groups.

7.5.1 RSA-measured change in femoral stem position in patients with high or low OB-capacity of their BM-MSCs

In patients with MSCs of low OB-capacity, the mean change in stem position along the y-axis (stem subsidence) at 3 and 24 months was more than twice that in patients with MSCs of high OB-capacity, although this difference did not reach statistical significance (**Table 7.15**). A similar trend was seen for the translation vector, representing the three-dimensional linear change in position (x, y and z axes). However, in the low OB-capacity group, stem subsidence at 3 months significantly exceeded the detection limit for stem migration along this axis (0.42 mm, p=0.020), which was not seen in the high OB-capacity group (p=0.351). Also for the translation vector, migration significantly exceeded the specific detection limit (0.40 mm) in the low (p=0.015), but not in the high (p=0.451) OB-capacity group. Change in stem position around the y-axis (stem rotation), as well as the three-dimensional rotational change in stem position (rotation vector), were of similar magnitude in the two OB-capacity groups (**Table 7.15**).

7.5.2 Cumulative stem migration after the 3 months settling period in patients with MSCs of high or low OB-capacity

In the whole group of 19 patients, the calculated cumulative migration from 3 to 24 months ranged between 0.1 to 2.2 mm (mean 0.6±0.5) along the y-axis (stem subsidence) and between 1.1 to 6.6 degrees (mean 3.0±1.5) rotation around the y-axis.

The magnitude of cumulative stem subsidence from 3 to 24 months was significantly higher in patients with MSCs of low in vitro OB-capacity (p=0.028) (**Figure 7.14A**), and the same was seen for total three-dimensional migration (translation vector, p=0.043) (**Figure 7.14B**). The cumulative rotation along the y-axis and three-dimensional rotation (vector) between 3 and 24 months varied widely between subjects, with corresponding magnitude in the two OB-capacity groups (**Figure 7.14C-D**). Importantly, differences in cumulative translational migration between patients with MSCs of high or low OB-capacity were not due to underlying demographic or clinical confounders, since there were no differences in these parameters between the two groups (**Table 7.15**). This was further checked separately for this subgroup of 19 patients using linear correlation analyses between the dependent variables (RSA) and possible confounders (demographics), confirming no interactions.

7.5.3 *In vitro* osteogenic capacity of MSCs as predictor of osseointegration

The time point for translational and rotational stem osseointegration was assessed for each individual patient based on translation and rotation vectors, respectively. Difference in osseointegration time point between patients with high and low in vitro OB-capacity of their MSCs was investigated with Kaplan-Meier time-to-event estimates, although the results should be interpreted with caution due to the low

number of patients. For translational osseointegration, the estimate was significantly different between the two groups (p=0.030). Patients with MSCs of high OB-capacity had a 43% probability of translational osseointegration within 3 months and a 100% probability of osseointegration within 6 months. In contrast, none of the patients with MSCs of low OB-capacity group showed translational osseointegration within 3 months, but had 73% probability of osseointegration within 6 months, and 91% probability of osseointegration within 12 months. There was no significant difference in estimates of rotational osseointegration. The estimated probability for osseointegration within 3 months was 57% in the high OB-capacity group and 25% in the low OB-capacity group.

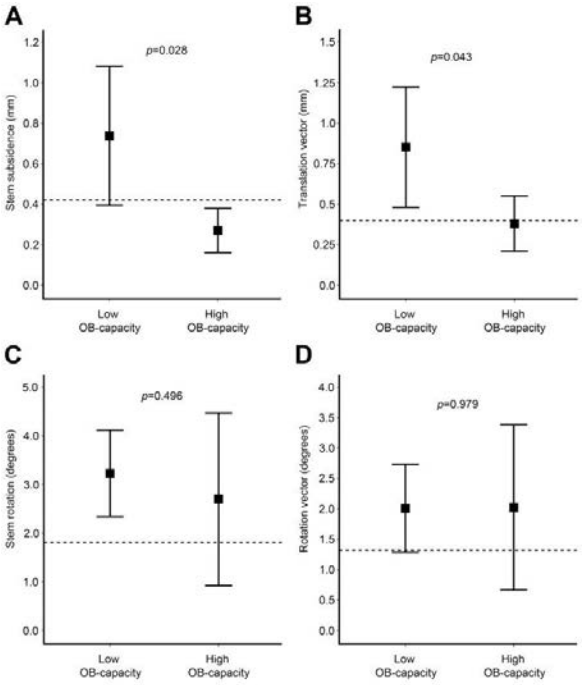

FIGURE 7.14 ***Cumulative stem migration from 3 to 24 months.*** *In patients with MSCs of low osteogenic capacity the magnitude of cumulative migration (A) along the y-axix (stem subsidence) and (B) in three-dimensional translation (translation vector) were significantly higher compared to the high OB-capacity group. (C) Rotational migration around the y-axis (stem rotation) and (D) three-dimensional rotation (rotation vector) were similar in the two OB-capacity groups. Mean with 95% CI. p=two-tailed p-value from Student's T-test. Dotted lines indicate parameter-specific detection limits.*

7.5.4 Clinical outcome in patients with high or low OB-capacity of their MSCs

RSA-measured stem micromigration had no impact on subjective or functional recovery from the hip replacement. There were no significant preoperative or postoperative differences in either WOMAC or HHS scores between the two groups (**Table 7.14**). Both groups showed a significant improvement in outcome measures postoperatively (p<0.001 for both). At 24 months, there were no signs of radiographic implant loosening or any other reason for revision surgery.

In one patient, the femoral stem had not yet stabilized by 24 months. In this patient, the total distance of translational migration from baseline to 24 months was 2.2 mm, and rotational migration was 4.4 degrees. There were no signs of stem loosening on plain radiographs, and the patient reported only minimal pain. This patient was osteopenic, had Dorr type A femur, showed signs of high bone turnover in the preoperative screening of serum markers, and was insufficient in 25(OH)D (43 nmol/l). The bone marrow MSCs of this patient showed slow growth rate, with only 1.1 PDs reached during passage 1. When culturing in osteogenic media cells were negative for both ALP and mineralization, despite repeated differentiation cultures (passage 2 and 3).

7.6 OFF-TRIAL THA PATIENTS ON OSTEOPOROSIS MEDICATION OR CORTICOSTEROIDS

Patients excluded from the main study due to ongoing bisphosphonate therapy (N=2), severe undiagnosed OP (T-score < -3.5) requiring initiation on bisphosphonate therapy (N=10), and patients who had ongoing corticosteroid treatment at baseline (N=4) were relocated into two off-trial subgroups that underwent the same study protocol as the study patients.

7.6.1 Preoperative patient characteristics (not previously reported)

The bisphosphonate subgroup (n=12) was slightly older (p=0.011) and had lower BMI (p=0.005) (**Table 7.16**) compared to the study patients (n=43). Serum markers of bone turnover and standard laboratory test all demonstrated similar levels in the off-trial subgroups compared to the study patients. The only exception was a higher level of S-25(OH)D in the corticosteroid subgroup (81±15 nmol/l) compared to the study population (58±21 nmol/l, p=0.045). Three patients in the bisphosphonate subgroup had vitamin D insufficiency (<50 nmol/l), and five patients had elevated PTH (>55 ng/l).

As expected, preoperative DXA measured T-scores were significantly lower in the bisphosphonate subgroup compared to the study population, even in the OA affected hip. However, the corticosteroid

subgroup displayed similar preoperative T-score levels as the study population (**Table 7.16**). Seven out of 12 patients in the bisphosphonate subgroup had Dorr Type C shape of their proximal femur, corresponding to a stove pipe geometry. Four patients had Type B, and only one patients had Type A. In the corticosteroid subgroup three out of four had Type A, and one had Type

TABLE 7.16 *Patient characteristics for off-trial subgroups*

	Bisphosphonate subgroup n=12	Corticosteroid subgroup n=4
Demographics (mean±SD, range)		
Age	71±8 (49 – 79)*	65±8 (56 – 74)^{NS}
BMI (kg/m^2)	27±4 (21 – 32)**	31±4 (27 – 36) ^{NS}
Years since menopause	23±8 (6 – 31)*	15±10 (1 – 26) ^{NS}
S-25(OH)D (nmol/l) (normal 50-140 nmol/l)	66±17 (41 – 90) ^{NS}	81±15 (71 – 103)*
S-PTH (ng/l) (normal 10-55ng/l)	50±27 (19 – 102) ^{NS}	42±12 (27 – 54) ^{NS}
Preoperative DXA measured T-scores (mean±SD, range)		
OA affected hip, neck	-0.75±1.16 (-2.84 to 1.50)*	0.09±2.30 (-1.80 to -3.10) ^{NS}
OA affected hip, trochanter	-1.56±0.88 (-3.05 to 0.40)***	0.43±1.70 (-1.80 to 1.80) ^{NS}
OA affected hip, total	-1.51±0.85 (-3.14 to 0.10)***	0.08±1.73 (-2.10 to 1.80) ^{NS}
Lowest T-score (10 areas)	-4.06±0.86 (-5.50 to -2.80)***	-1.59±1.20 (-2.90 to 0.00) ^{NS}
Highest T-score (10 areas)	-0.441±0.87 (-1.40 to 1.50)***	0.86±1.98 (-1.50 to 3.10) ^{NS}
Frequencies, n		
Postmenopausal	12	4
Use of estrogen replacement	0	1
Use of calcium supplement	6	3
Use of vitamin D supplement	7	2
Previous fractures	7	2
S-25(OH)D insufficiency (≤ 50nmol/l)	3	0
S-PTH elevated (> 55 ng/l)	5	0
Smoking	1	0
Alcohol consumption >3 drinks/week	1	0
Dorr classification		
Type A	1	3
Type B	4	0
Type C	7	1

Comparisons between the off-trial subgroups and the main study population (N=43) with Mann-Whitney U test; *p<0.05; **p<0.01; ***p<0.001; NS=not statistically significant

7.6.2 Quality of intertrochanteric cancellous bone (not previously reported)

Unsurprisingly, the quality of intertrochanteric cancellous bone biopsies from the bisphosphonate subgroup was significantly decreased. Structural parameters obtained from μCT data revealed significantly lower bone volume (BV/TV 11±3%), decreased trabecular thickness (Tb.Th 0.16±0.01 mm) and decreased trabecular number (Tb.N 0.66±0.16/mm) of the cancellous bone biopsies obtained from the bisphosphonate subgroup compared to the study patients (BV/TV 15±5%, p=0.002; Tb.Th 0.18±0.03 mm, p=0.001; Tb.N 0.80±0.23, p=0.023, respectively). Despite large individual variability in biomechanical properties, cancellous bone biopsies from the bisphosphonate subgroup showed significantly lower ultimate compression failure force (10.4±9.4 N), and lower stiffness (13.4±13.1 N/mm) compared to bone biopsies from study patients (17.5±15.7 N, p=0.050; 35±47 N/mm, p=0.014). There were no differences in any of the μCT derived microstructural properties or biomechanical parameters in the corticosteroid subgroup compared to the study patients.

7.6.3 In vitro properties of bone marrow MSCs (not previously reported)

A bone marrow aspirate was obtained from all off-trial patients. From two patients (bisphosphonate subgroup) the MNC yield was low, resulting in few growing cells and samples were excluded from the analyses. From the remaining bone marrow aspirates the yield of MNCs and subsequently MSCs were similar to that of the study population. Growth kinetics of MSCs varied widely within the off-trial group, but fell within the same ranges as the primary study population. There were no differences in proliferative capacity (Max PDs) or proliferation rate (Max PD rate) compared to the study population (**Table 7.17**).

The osteogenic capacity for MSCs from patients of the off-trial subgroups was significantly decreased. For MSCs from the bisphosphonate subgroup, OB differentiation capacity, assessed by staining for ALP (N=9), was on average 50% lower compared to MSCs from the study population (p=0.031). For MSCs from the corticosteroid subgroup (N=4) OB differentiation was negligible, with average ALP stained area of only 5% (range 2-8%) and no mineralization detected for MSCs of any the four patients. Also in the bisphosphonate subgroup a majority of the MSCs failed to fully differentiate into mineral producing OBs. From six of the nine patients included in the osteogenic assays, no mineralization was achieved despite repeated osteogenic culturing at several passages (p1-3).

7.6.4 Two-year clinical follow-up

In the off-trial group, one patient had revision surgery during follow-up due to periprosthetic fracture and one was excluded from the RSA follow-up due to absence of bone markers. Both were from the bisphosphonate subgroup.

7.6.4.1 Disease scores of functional outcome of the THA (not previously reported)

Subjective evaluation of functional outcome of the THA (N=16) based on disease scores demonstrated significant improvement at the same level as in the study population. Harris hip score improved from 51 (range 24-82) preoperatively to 81 (range 38-97) by 3 months ($p<0.001$) and 86 (range 63-98) by 24 months ($p<0.001$). Correspondingly, WOMAC scores improved from 47 (range 16-90) preoperatively to 22 (range 4-67) at 3 months ($p<0.001$), and 14 (range 0-35) by 24 months ($p<0.001$). Two patient reported moderate pain at 24 months, while all other had minimal or no pain. According to the total scores, all patients reported minimal or mild symptoms at 24 months.

7.6.4.2 Stem migration and osseointegration

Stem migration followed same pattern as in the study population. There were no statistically significant differences in average stem migration in any RSA parameter (y translation, translation vector, y rotation, rotation vector) between the off-trial subgroups and the study patients. In the corticosteroid subgroup all stems had stabilized by 6 months, suggesting there was no apparent influence of corticosteroids on stem osseointegration. In the bisphosphonate subgroup, seven out of ten patients showed osseointegration within 6 months. In one patient, the stem had not yet stabilized by 24 months. This patient (initiated on OP medication at time of surgery) had stem rotation of 3.1 degrees by 12 months with an additional 0.5 degrees rotation by 24 months. The patient reported moderate pain at 24 months.

TABLE 7.17 BM-*MSC s from off-trial subgroups, mean ±SD (range)*

	Bisphosphonate subgroup n=10	Corticosteroid subgroup n=4
Bone marrow aspirate (ml)	4.8 (3.5 – 7)	5.0 (4 – 6)
MNCs/ml bone marrow	2.0 x 10^6 (0.1 – 8.6 x 10^6)	4.3 x 10^6 (1.5– 9.9 x 10^6)
MSCs p0/10^3 MNC plated [a]	105 (20 – 192)	12 (125 – 305)
CFU/BM MNCs (%)	0.005 ± 0.004	0.008 ± 0.003
Max PDs	3.7 (3.2 – 4.7)	4.0 (2.1 – 6.2)
Max PD-rate (PDs/day)	0.12 (0.06 – 0.22)	0.10 (0.02 – 0.18)
ALP stained area (%)	#25 (6 – 70)*	5 (2 – 8)**
von Kossa stained area (%)	&10 (0 – 56)[a] 29 (2 – 56)[b]	0 –

n=9 ; & n=9; [a]All donors, including zero values; [b]Only values >0 included (n=3); asterisk indicate $p<0.05$ compared to the main study population using Mann-Whitney U test

7.6.4.3 Periprosthetic bone loss (not previously reported)

In the corticosteroid subgroup, the periprosthetic BMD or BMD changes over time did not differ from the study patients. In the bisphosphonate subgroup however, there were clear differences compared to the study population.

The bisphosphonate subgroup had significantly lower periprosthetic BMD compared to the study patients both at baseline and at all follow-up time points. This was seen in all Gruen zones (G1-G7) as well as in the total periprosthetic area (p-values between <0.001 and 0.034). In the bisphosphonate subgroup, total periprosthetic BMD (Gruen zones 1-7) was on average 1.04±0.09 g/cm^2 at baseline compared to 1.23±0.11 g/cm^2 in the study population ($p<0.001$), with a similar difference maintained up to 24 months.

However, analyzing the changes in periprosthetic BMD over time displayed different patterns in the two groups, with less bone loss in the bisphosphonate subgroup compared to the study patients, probably reflecting the influence of the medical therapy. While the study population exhibited significant early periprosthetic bone loss in all Gruen zones including the total area, except for G4 and G5, the bisphosphonate subgroup showed significant bone loss only in Gruen zone 7 corresponding to calcar rounding following THA. In Gruen zones 2, 3, 4, 6, and the total area, periprosthetic BMD remained unchanged compared to baseline in the bisphosphonate subgroup. In Gruen zone 5, the same pattern of significant bone gain presented by the study patients was seen for the bisphosphonate subgroup. In contrast, the bisphosphonate subgroup showed a significant bone gain also in Gruen zone 1, whereas this zone was associated with significant early (3 months) bone loss in the study population.

8 DISCUSSION

Long-term success of cementless THA relies on proper biological fixation of the implant to the surrounding bone, a process assumed to take 3-6 months. Aside from appropriate implant properties, bone quality and osteogenic potential at the implantation site are considered to dictate the course of osseointegration (Engh et al., 1987, Khanuja et al., 2011), but very little is known about factors actually affecting the process of osseointegration. Cementless fixation represents over 50% of all primary THAs in most of the Western world (Carli and Jerabek, 2015, Sadoghi et al., 2012), and postmenopausal women with primary hip OA constitute a majority of these patents (Mäkelä et al., 2008, Sadoghi et al., 2012). Still, their systemic bone quality status is rarely examined prior to THA, although age-related decrease in bone quality and MSC function can contribute to impaired bone healing and delayed osseointegration. Early implant migration and osseointegration of cementless femoral stems has never been prospectively monitored in postmenopausal women at risk of decreased systemic bone quality. Considering the essential role of MSCs in the process of osseointegration, it is important to better understand the relationship between osteogenic properties of MSCs and implant healing.

8.1 DISCUSSION ON RESULTS

8.1.1 Bone quality in postmenopausal women scheduled for cementless THA

Systemic BMD

The prevalence of decreased systemic BMD was high in the female patients scheduled for cementless THA, as the majority of the patients (39 out of 53, 74%) presented with undiagnosed osteopenia (45%) or OP (28%). Low systemic BMD was associated with signs of increased bone turnover as assessed by serum bone metabolic markers (OCN, PINP, NTX). Patients with OP were older and had lower BMI. In addition, abnormal laboratory results were found in an unexpectedly high number of patients (n=8, 15%), requiring preoperative consultation with an endocrinologist and postponing of the surgery. Five patients (9%) were diagnosed with secondary OP. These findings correspond well with those of Glowacki and co-workers (2003). In their preoperative analysis of a similar group of 68 postmenopausal women (aged 59-74 yrs) scheduled for THA, they found occult OP in 25% of the patients. Also in their study, markers of bone turnover (OCN, ALP, NTX) were elevated in the osteoporotic patients, who were older, had lighter weight and higher number of years since menopause compared to the hip OA patients with normal BMD.

The co-existence of hip OA and OP is not a new finding within orthopaedics (Healey et al., 1985, Dorr et al., 1990, Karvonen et al., 1998). Still, the original idea of an inverse relationship dominated at the time of initiation of the current project, and there were little indications supporting screening for decreased BMD in the general postmenopausal female patient with hip OA. Several studies have confirmed our results. In patients with hip or knee OA scheduled for joint replacement, Breijawi *et al* (2009) found high prevalence of osteopenia (43%) and OP (23%) among the female patients (age 70, 50-83 yrs). Low BMD was associated with higher age and lower BMI. Corresponding frequencies (43% osteopenia, 23% OP) were found in a population of female and male (43%) patients with severe hip or knee OA (Lingard et al., 2010), and in 13 male and 16 female hip OA patients 38% had osteopenia and 21% had OP (Domingues et al., 2015). In a population of 214 pre- and postmenopausal females (age 65±11) with mixed arthritic etiology 35% were found to have OP (Labuda et al., 2008).

The current study, together with previous and more recent studies clearly demonstrate that hip OA does not protect female patients from OP. On the contrary, the observed prevalence corresponds to expected frequency in the general population according to age and gender, as reported for studies from Sweden (Kanis and Gluer, 2000), Canada (Sawka et al., 2006), and for several European countries, Australia and USA (Sanchez-Riera et al., 2014, Napoli et al., 2014).

Vitamin D status

Applying a threshold level of 50 nmol/l (20 ng/ml) for serum 25(OH)D, vitamin D deficiency was found in 19 (36%) of the patients. This is a higher prevalence compared to the 22% vitamin D deficient reported for the corresponding patient group in the study by Glowacki *et al* (2003). However, in their study a cut-off level of 37 nmol/l (18 ng/ml) was used. In the present study, seven patients (13%) had serum vitamin D levels <37 nmol/l. The lack of established cut-off levels for defying deficiency/insufficiency makes comparisons of results between studies difficult. The 75 nmol/l (30 ng/ml) is a widely used cut-off for deficiency, and is also the threshold recommended by the International Osteoporosis Foundation (Dawson-Hughes et al., 2010). In a worldwide study of postmenopausal women with OP, 64% had vitamin D lower than 75 nmol/l (30 ng/ml)(Lips et al., 2006). In the current study, 77% of the THA patients had serum vitamin D below 75 nmol/l, which corresponds to the frequency reported by Napoli *et al* (2014). This is a slightly lower

compared to the 86% reported for postmenopausal females with hip OA (Breijawi et al., 2009), and 83% reported for male and female patients with hip or knee OA (Domingues et al., 2015). The mean level of serum 25(OH)D in the present study (59±20 nmol/l) is within the same range (45-55 nmol/l) as seen in other studies on postmenopausal female OA patients (Glowacki et al., 2003, Breijawi et al., 2009, Domingues et al., 2015), general population of postmenopausal women (Napoli et al., 2014) and postmenopausal women with OP (Lips et al., 2006).

Serum levels of vitamin D decreases with aging and ensuring adequate levels is important for maintaining optimal musculoskeletal health, especially in postmenopausal women where deficiency can aggravate OP (Rizzoli et al., 2014). Vitamin D is also important for maintenance of a functional MSC pool. In the general population low serum vitamin D correlate with decreasing BMD, and supplementation reduces bone loss and fracture risk. This seem not to be the case in postmenopausal women, where serum 25(OH)D appear independent of BMD status (Lumachi et al., 2013). In the present study, no correlation was found between BMD and vitamin D status. This finding confirms previous observations (Glowacki et al., 2003), and is further supported by similar results in later studies (Breijawi et al., 2009, Domingues et al., 2015, Lumachi et al., 2013). The more homogenous patient populations in these studies compared to the general population can explain the lack of correlation, but probably also reflect the multifactorial nature of OP.

The local bone quality in OA affected hip

In cementless THA, properties of the local bone of the hip and proximal femur have impact on both immediate and long-term stability of the femoral component. In this thesis, the investigated properties of the local bone included standard DXA measurements of the hip, geometrical shape of the femur, as well as microarchitectural and biomechanical properties of the intertrochanteric cancellous bone.

DXA

In the OA affected hips, the projected area and the BMC of the femoral necks were increased whereas the BMC at the trochanter was reduced compared to the contralateral side. No differences were found in the total hip values, nor were there any differences in BMD in any of the regions (femoral neck, trochanter, total hip) compared to the contralateral side. This is in line with other DXA studies indicating that hip OA is associated with changes predominantly at the neck and trochanter of the affected hip, but not necessarily at total hip, contralateral hip or remote skeletal sites (Arokoski et al., 2002a, Sandini et al., 2005, Wolf et al., 2009, Glowacki et al., 2010). Although we did not see a difference in BMD, increased femoral neck but not total hip BMD compared to the contralateral hip have been reported for patients with unilateral hip OA (Arokoski et al., 2002, Glowacki et al., 2010). In contrast to several other reports, Wolf *et al* (2009) found reduced total hip BMD and BMC of the affected hip compared to the contralateral side. Compared to control subjects, total hip BMD seem unaltered in hip and knee OA patients (Stewart et al., 1999, Ding et al., 2010, Arokoski et al., 2002a, Sandini et al., 2005), although hip OA seems to be associated with increased BMD of the femoral neck (Antoniades et al., 2000, Burger et al., 1996). In a multicenter cohort study of 5,245 women, increased area of the femoral neck was associated with developmental stages of hip OA, and re-distribution of proximal femur bone mass was observed early in the disease (Javaid et al., 2009).

Whether hip OA is associated with increased hip BMD has been subject of debate. Aside from the apparent differences depending on sub regions, a number of factors influence the BMD measurements. First, BMD depend on the type of joint changes present (Antoniades et al., 2000), and changes in DXA outcome correlate with severity of the OA (Nevitt et al., 1995, Bruno et al., 1999, Arokoski et al., 2002a). Further, suboptimal positioning of the hip during DXA scanning lead to overestimation of BMD (Wolf et al., 2009). Detection of significant changes vary depending on the comparison; the contralateral side or control subjects.

The mechanisms behind DXA measured changes of the proximal femur in OA patients are unclear. Local changes can be part of hip OA pathogenesis affecting both cartilage and adjacent bone (Nevitt et al., 1995, Antoniades et al., 2000, Javaid et al., 2009). It is possible that re-distribution of BMD in the subregions reflect altered loading conditions due to decreased weight bearing and limited range of motion (Burr, 1997, Arokoski et al., 2002b, Moisio et al., 2004). The consequences seem to be decreased risk of femoral neck fractures (Dequeker and Johnell, 1993), which partly explains the idea of inverse relationship between hip OA and OP, and increased risk of trochanteric fractures (Middleton and Ferris, 1996, Calderazzi et al., 2014). Another consequence of increased femoral neck BMD in hip OA is the general overestimation of systemic bone quality in these patients (Glowacki et al., 2010).

MICOARCHITECTURE

The quality of the intertrochanteric cancellous bone varied widely among the patients. Decrease in biomechanical properties and trabecular thickness correlated with increasing age. This is in line with well-known age-related changes in cancellous bone structure (Djuric et al., 2010, Gabet and Bab, 2011) and mechanical properties (Tjhia et al., 2012).

The trabecular microstructure vary considerably between skeletal sites, but also within skeletal sites, especially at sites subjected to large load-bearing (Parkinson and Fazzalari, 2013). In addition, age-related changes in cancellous bone

structure are gender- and site-dependent (Djuric et al., 2010, Shanbhogue et al., 2016). This makes comparisons to previous reports difficult, since most studies have focused on changes in cancellous bone in femoral head, femoral neck, iliac crest or vertebral bodies. Data available on changes in cancellous bone microarchitecture in the proximal femur is limited.

Despite variability depending on age, gender and skeletal site, reduction in trabecular number with subsequent loss of connectivity are the most distinct age-related changes in cancellous bone (Gabet and Bab, 2011), and seem to be independent of the skeletal location. In the current study, patients with low systemic BMD displayed decreased microarchiterctural and biomechanical properties of their intertrochanteric cancellous bone. In addition to increase in trabecular pattern factor (Tb.Pf) as a sign of decreased connectivity, patients with low systemic BMD also displayed a trend towards decreased bone volume (BV/TV) and trabecular number (Tb.N), and increased trabecular separation (Tb.Sp). This trend of different spatial distribution of trabeculae in patients with low systemic BMD compared to patients with normal systemic BMD fits well with previous studies of cancellous bone from femoral head (Li and Aspden, 1997, Ciarelli et al., 2000) and femoral neck (Blain et al., 2008) indicating loss of bone volume, decreased trabecular number and loss of connectivity in women with hip fractures compared to controls and women with OA. The fact that all patients in the current study had hip OA makes our results interesting. Most reports on the subject has compared cancellous bone microarchitecture between subjects with OA and OP, and describes differences. Aside from the expected OA related changes in DXA measured parameters in the femoral neck and the trochanter, i.e. in the subregions closest to the affected area, the intertrochanteric cancellous bone structure seem to be more affected by the systemic bone health status than the local OA.

PROXIMAL FEMORAL CANAL GEOMETRY

The shape of the femoral canal was not only age related but correlated also with the systemic BMD status. While Dorr type C femurs were only found in hip OA women with concomitant OP, unaltered Dorr type A femurs were predominantly found in patients with normal systemic BMD. Of the 25 patients with Dorr type B femurs, only 3 had normal systemic BMD, while most had osteopenia (n=15) and seven had OP. Considering the well documented changes in cortical bone tissue in women following aging, menopause and OP (Väänänen and Härkönen, 1996, Zebaze et al., 2010) these findings are not surprising. However, specific research focus on the relationship between deterioration of the femoral canal shape and OP (and osteopenia), especially in postmenopausal women with hip OA, is restricted. Sha *et al* (2007) investigated how radiographic measures of the proximal femur and Dorr type correlated with occult OP in postmenopausal women with hip OA, and found that Dorr classification correlated with DXA measured T-scores. This corresponds to the findings in the current thesis study.

Also the CFI followed the systemic BMD status, with low index (<3) indicating straight stove pipe shaped femurs only in osteoporotic and osteopenic patients, and high index (>4.7) corresponding to tapered shaped femurs only in patients with normal systemic BMD. In the study by Sah *et al* (2007), no correlation was found between T-score and the canal-to-calcar ratio. One explanation to the discrepancy in outcomes is that in calculation of canal-to-calcar ratio the intramedullary width is measured at the fixed point 10 cm below mid-lower trochanter irrespective of femoral length, while in CFI calculation intramedullary width is measured using the femoral isthmus as an anatomical landmark, accounting for differences in patient height.

The osteogenic capacity of bone marrow MSCS from postmenopausal THA patients

The yield of MSCs from THA patients was within the expected frequencies according to donor age and profile, as previously reviewed elsewhere (Alm et al., 2014). Of interest in the context of cementless THA is the lower number of available bone marrow MSCs in bone marrow of THA females compared to the younger reference group, indicating a decreased reservoir of MSCs. This confirms previous reports on decreased yield of bone marrow MSCs from female THA patients compared to younger (Garvin et al., 2007), and male patients (McCann et al., 2010). The proliferative capacity and doubling rate varied widely and were generally low, although within reported ranges for MSCs from comparable patient groups (Stenderup et al., 2003).

There were large individual variations in the *in vitro* osteogenic differentiation capacity as analyzed by ALP activity and ALP staining, although MSCs from all patients did display some degree of differentiation. In contrast, MSCs from all patients did not display mineralization. Since the mineralization assay was repeated with MSCs from these 13 patients at several occasions and at different passages, the outcome is truly negative and not related to experimental differences. Due to the design of the current study the underlying mechanisms for failed mineralization were not investigated in further detail. Based on the established literature, it is not surprising that MSCs from this patient group displayed low differentiation and mineralization *in vitro*. It seems as both OA and OP have adverse effects on *in vitro* properties of human MSCs, although the type and magnitude of these effects are inconclusive. MSCs isolated from osteoporotic subjects have shown a decreased *in vitro* osteogenic capacity (Rodriguez et al., 1999, Zhang et

al., 2009), while the effect of OA on MSCs seems unaltered in comparison (Murphy et al., 2002, Jones et al., 2010, Zhang et al., 2009), although changes in other properties have been observed (Mwale et al., 2011, Rollin et al., 2008b, Jiang et al., 2011). Reduced mineralization has been reported for MSCs from patients comparable to the current study (Rodriguez et al., 1999, Zhang et al., 2009). The current study was not designed to evaluate the effect of OP and OA on MSC capacity. Comparisons to MSCs of the reference group was primarily aimed to ensure that possible decreased capacity of MSC from THA patients was not due to suboptimal protocols, and secondarily as an indication for functionality of MSCs from THA females. The expansion capacity, osteogenic differentiation and mineralization properties of MSCs from the postmenopausal THA females were approximately 50% compared to MSCs from the reference group of younger premenopausal females (mean age 40±16, range 19-60) from our previous study (Alm et al., 2010).

The primary aim was not to investigate correlations between MSC properties and demographic parameters; rather this was performed as secondary, *post hoc*, analyses including all available MSCs (n=30). Correlations found were not strong, but yet statistically significant. Our results are in line with established observations of associations between *in vitro* osteogenic differentiation of MSCs and bone quality parameters of the subjects. MSCs from patients with higher systemic BMD and higher bone turnover showed increased osteogenic differentiation capacity. The correlation of ALP expression with preoperative T-scores and inverse correlation with serum vitamin D corresponds to results by Zhou *et al* (2012). Their detailed analyses revealed an increased *in vitro* response of MSCs from vitamin D deficient patients to osteogenic culture conditions, explaining the inverse relationship. In contrast to their study, we did not find a correlation between serum PTH levels and osteogenic differentiation of MSCs, which could be explained by the higher PTH levels in our study.

8.1.2 An optimized *in vitro* osteogenic differentiation assay for human MSCs

Several promising MSC applications would benefit from an *in vitro* protocol ensuing terminal osteoblastic differentiation, independently of donor-related varieties. With the aim of developing such a protocol, enhanced differentiation and mineralization was found when using transient treatment with 100 nM Dex for the first week of induction culture. This treatment decreased the variability in osteoblastic characteristics between MSC from different individuals. Transient treatment with 10 nM Dex was not as effective as 100 nM.

The initial experiments indicated that presence of Dex during the first week of induction is critical for proper osteoblastic differentiation. This is reasonable since phenotype commitment seem to be determined early in the culture period (Jaiswal et al., 1997), and an early report suggested that 7 days supplementation with Dex is enough for terminal osteoblastic differentiation (Cheng et al., 1994). Previous studies support our results on the long-term stimulatory effects of transient Dex treatment on osteogenic differentiation (Cheng et al., 1994, Fromigue et al., 1997, Schecroun and Delloye, 2003).

Controversies regarding the use of Dex for *in vitro* differentiation of human MSCs often relates to adverse effects of long term *in vivo* use of GCs (Hardy and Cooper, 2011, Canalis, 2005). *In vitro* Dex is known to inhibit cell proliferation (Cheng et al., 1994, Walsh et al., 2001, Kim et al., 1999). Also in the current study significantly delayed proliferation was found with continuous Dex treatments, but in contrast transiently treated cultures showed proliferation equal to control cultures. Our results are in agreement with previous studies (Jaiswal et al., 1997, Jorgensen et al., 2004), but in conflict with results by Cheng *et al* (1994) who found that both transient and continuous Dex treatment inhibit proliferation. The response to Dex depends not only on the concentration and duration of supplementation, but on the commitment and differentiation stage of the cells (Fromigue et al., 1997, Xiao et al., 2010, Hardy and Cooper, 2011), which can explain some of the discrepancies.

Dex is suggested to inhibit or postpone COL1 (Fromigue et al., 1997) and reduce OCN production (Jorgensen et al., 2004). We found positive COL1 staining already at 14 days with transient Dex treatment. In contrast to earlier proposals (Jorgensen et al., 2004), OCN was not detected in cultures without Dex, while transiently Dex-treated cultures showed uniform and widespread OCN staining. Also continuously treated cultures were positive for OCN, which is in line with previous reports (Cheng et al., 1994, Pei et al., 2003). The methods applied in our study did not allow assessing the amount and distribution of collagenous matrix in different culture conditions. It is likely that the different morphologies seen with different Dex treatments influence the distribution of the collagen matrix, and subsequently the 3D structure of the mineralized matrix. MSCs undergo morphological changes upon differentiation, and the changes in cytoskeletal organization are critical for osteogenic maturation (Rodriguez et al., 2004, Mauney and Volloch, 2009). Dex induced upregulation of transcriptional co-factor FHL2 upregulate COL1 expression (Hamidouche et al., 2008). Further, Dex induced modulation of Runx2 phosphorylation by upregulation of MKP-1 has been demonstrated to be crucial for expression of OCN and

mineral deposition (Phillips et al., 2006). These effects of Dex can be part of the underlying mechanisms for the different 3D structures, as well as COL1 and OCN staining patterns observed with different Dex treatments.

There is a recognized donor-to-donor variation in human MSCs and even variations between experiments with cells from the same donor, as extensively reviewed (Bara et al., 2014). Both donor characteristics and the stage of the MSC are known to affect the response to Dex (Beresford et al., 1993, Hung et al., 2006, Xiao et al., 2010). If culture conditions are suboptimal, variations might be greater due to differences in responsiveness among heterogenic donor populations. As an important advantage, the current study demonstrated smaller inter- and intra-individual variations with transient Dex treatment. Despite heterogeneity in donor characteristics (age, hip OA), transient Dex treatment showed only minor individual variations, whereas continuous or no Dex treatment resulted in higher variations. Another crucial observation from the current study was that despite of age-related differences in the level of osteoblastic differentiation, transient treatment with 100 nM Dex provided best outcome in all age groups. Notably, all donors in this study were females. However, the effect of gender on the MSC responsiveness to osteoblastic induction and Dex seems secondary to age and other demographic factors (Hung et al., 2006, Siddappa et al., 2007). Although gender of the donors are not always specified in previous investigations on Dex-response in human MSCs, studies addressing the issue have reported no gender-related difference (Walsh et al., 2001, Siddappa et al., 2007, Mendes et al., 2004, Anselme et al., 2002). Therefore it is reasonable to assume that transient 100 nM Dex treatment provide high degree of differentiation and low variation also with MSC from male donors.

The increased understanding how Dex exerts osteogenic effects on MSCs has revealed several mechanisms through which Dex induces and regulates RUNX2 (Hamidouche et al., 2008, Hong et al., 2009, Phillips et al., 2006). These mechanisms can explain the long-term stimulatory effect of transient Dex treatment observed in the current study. The seven day Dex treatment ensured osteogenic commitment of the MSCs, and once the transcriptional program is turned on, differentiation is in progress. These transcriptional actions of Dex can also explain the decreased variability with transient treatment, since prolonged supplementation will target cells at different stages, accounting for increased variability between donors and experiments.

8.1.3 Periprosthetic bone remodeling - influence of systemic BMD

In ***Study III***, monitoring of periprosthetic bone remodeling showed only temporary loss of total periprosthetic BMD during the first 12 months, followed by recovery. At 24 months, statistically significant bone loss was seen only in the proximal Gruen zone 7. Low systemic BMD (osteopenia and OP) predicted this adverse local remodeling, while neither local preoperative BMD of the operated hip nor baseline BMD of Gruen zone 7 were significant predictors of the process.

The low total periprosthetic bone loss in the present study, reaching a maximum value of only 3.8% at 6 months followed by recovery approaching baseline by 2 years, is slightly less than other studies. The total periprosthetic BMD is reported to decrease by 5-10% during the first 2 years after cementless THA (Venesmaa et al., 2001, Wolf et al., 2010, Lazarinis et al., 2013, Flatoy et al., 2016), depending on the femoral stem design, with slow progressive loss or minimal recovery during the following years (Panisello et al., 2009b, Aguilar Ezquerra et al., 2016). The maintenance of periprosthetic bone seen in the current study most likely reflects the mechanical characteristics of the femoral stem (ABG II), designed to re-establish physiological load as far as possible in order to avoid stress-shielding (van Rietbergen and Huiskes, 2001, Gracia et al., 2010). In previous studies using the same implant, the 2-year decrease in total periprosthetic BMD have been 4.1% (Van der Wal et al., 2006). Although a larger stem size can be associated with increased periprosthetic bone loss (Nishii et al., 1997, Skoldenberg et al., 2006), this is not necessarily the case with the ABG II stem. With larger stems, initial stability can be improved and the distal bone loss seen with smaller ABG II stems can be avoided (Van der Wal et al., 2006). Larger stems may also contribute to prevention of malalignment, which seems to increase periprosthetic bone loss in ABG II arthroplasties (Panisello et al., 2006).

Major bone loss in the proximal calcar area (zones 1 and/or 7) is a common feature seen with both cemented and cementless THA. In the current study, major bone loss was seen in Gruen zone 7. The decrease in BMD was 16% at 3 months, 21% at 12 months and 23% at 24 months, which was within the 12 (12 to 31%) and 24 months (14 to 28%) ranges reported for cementless stems of different designs (Venesmaa et al., 2001, Rahmy et al., 2004, Boe et al., 2011a, Lazarinis et al., 2013). The bone loss seen in Gruen zone 7 is somewhat higher compared to other studies using the same implant (Van der Wal et al., 2006, van der Wal et al., 2008, Panisello et al., 2006, Aguilar Ezquerra et al., 2016). This can, at least partly, be explained by differences in patient groups. In addition to heterogeneous etiology, other studies include male patients. In addition, our patient group

included patients with low systemic BMD. In patients with normal systemic BMD, calcar bone loss at 1 year (12.5%) and 2 years (16%) corresponded to that reported in other studies on the same stem (Panisello et al., 2006, Panisello et al., 2009b, Aguilar Ezquerra et al., 2016). Extending previous observations (Rahmy et al., 2004, Grochola et al., 2008, van der Wal et al., 2008, Arabmotlagh et al., 2005), the current study demonstrated increased bone loss in Gruen zone 7 in patients with low systemic BMD.

In contrast to other implant designs or other type of patients (Venesmaa et al., 2001, Skoldenberg et al., 2011b, Aguilar Ezquerra et al., 2016), periprosthetic bone loss did not correlate with the local preoperative BMD of the operated hip or baseline periprosthetic BMD in the current study. This is most likely due to the secondary changes of BMD at femoral neck and trochanter caused by the underlying OA (***Study I***). This study further demonstrated that four metabolic bone markers and the uncoupling index predicted the temporary changes in zones 2 and 5 during the first 3-6 months. This indication for high turnover in correlation with periprosthetic bone remodeling probably reflects the healing process of cementless THA, which is known to mimic fracture healing. Not many have applied bone metabolic markers for monitoring periprosthetic bone loss in cementless THA, and the predictive value is unclear (Wilkinson et al., 2003, Yamaguchi et al., 2003, Habermann et al., 2007, Kenanidis et al., 2010).

Our results support the idea that implant-related factors are most critical for preserving the total periprosthetic bone stock (van Rietbergen and Huiskes, 2001). As previously reported for the same (Panisello et al., 2006, Panisello et al., 2009b) and for other stem types (Gibbons et al., 2001, Brodner et al., 2004), loss of bone stock in the calcar region remain despite modifications towards bone preserving designs. It appears that if implant related parameters known to affect periprosthetic bone loss are controlled for, the systemic bone metabolic status has more influence than local bone parameters. This holds true at least with this particular femoral stem in postmenopausal women with hip OA. The long-term effects of increased calcar bone loss in patients with low systemic BMD needs to be evaluated. Some evidences indicate an increased risk of late-occurring fractures with loss of proximal bone stock in certain patient groups (Hsieh et al., 2005, Streit et al., 2011, Skoldenberg et al., 2014).

8.1.4 Stem migration and osseointegration

Previous RSA studies have shown that cementless stems of different designs show minimal or no subsidence (**Table 2.3**) or rotation in younger and middle-aged patient populations of mixed gender, while RSA studies of cementless femoral stems in aged and osteoporotic patients are scarce. The magnitude and duration of early stem migration varies with stem design, but the common feature is subsidence during the first 3-6 months when the stem settles, while marginal subsidence is detected thereafter (**Table 2.3**). But there are cementless stem designs with warning signs of continuous migration suggesting insufficient primary stability, as detected by RSA (Luites et al., 2006, Simpson et al., 2010, Baad-Hansen et al., 2011) and by EBRA (Friedl et al., 2009a, White et al., 2012).

Separate parameters affect initial and prolonged micromigration of cementless femoral stems. It also appears that subsidence and rotation of the stem are influenced by separate parameters. The magnitude of rotational migration was slightly larger in this study compared to RSA studies of other cementless stem designs. Rotational migration was detected across the sub groups, and neither systemic BMD status nor osteogenic capacity of MSCs predicted the magnitude of rotational migration or time point for rotational stabilization. Aging and widening of the femoral canal were however risk factors for delayed rotational stability, indicating rotational migration observed in the study is related to the stem design.

Due to differences in migration patterns with stem design (**Table 2.3**) it is difficult to estimate how much of the associations of stem migration and osseointegration with systemic BMD and MSC-capacity are related to the specific implant used, since 1) the ABG-II stem has not been evaluated with RSA before, 2) cementless femoral stems have not been monitored with RSA in this type of patient group before with regards to age and systemic bone quality, and 3) cementless stem osseointegration has never been compared to *in vitro* osteogenic capacity of patients' MSCs before.

THE SIGNIFICANCE OF SYSTEMIC BMD

Postmenopausal females with a high incidence of undiagnosed OP are at risk of increased migration of their cementless stems and delayed osseointegration. The average degree of subsidence was minimal (0.5 mm, 0.0 – 2.0) in patients with normal BMD, while patients with low BMD showed two-fold (1.3 mm, range 0.0 – 1.2) subsidence. These values do not represent the true worst-case scenario since the patients with undiagnosed severe OP were excluded. The average subsidence in patients with normal systemic BMD corresponds to those reported for several other stem designs in middle-aged patient populations (Bottner et al., 2005, Luites et al., 2006, Ström et al., 2006, Lindalen et al., 2012) and in a population of corresponding age range (Campbell et al., 2011).

The increased subsidence observed in patients with low systemic BMD corresponds to migration observed in younger and middle-aged patients upon full weight-bearing (Bottner et al., 2005, Ström et al.,

2007), and for a stem without HA coating that was subsequently discontinued (Luites et al., 2006)(**Table 2.3**). As reported by two independent research groups (Simpson et al., 2010, Weber et al., 2014), the Furlong Active stem display corresponding magnitude and delayed pattern of migration as in the low BMD patients of the current study. In both studies, the age of the patients were corresponding to our study. Despite increased early migration, no additional migration was observed the following 1-5 years (Weber et al., 2014). In a series of 30 patients (9 male and 18 female) with a median age of 70 years, Campbell and co-workers (2011) recognized a striking variability in the magnitude of subsidence within the first six months which they assumed to be related to differences in quality of initial fixation or bone quality. Considering the minimal amount of stem subsidence reported with straight or custom-made stems in younger patients in previous studies (Table 2.3), and our results, it is likely that the variability observed by Campbell and co-workers (2011) is due to differences in systemic BMD and quality of the local bone stock. A 6-year follow-up demonstrated no additional migration between 2-6 years (Callary et al., 2012). Supporting our results, the recently reported migration for an ultra-short and a conventional stem in patients aged 62±5 years, of which 34% had low BMD (total hip and lumbar spine), were corresponding to the magnitudes observed in our patients with low and normal BMD, respectively (Salemyr et al., 2015).

THE SIGNIFICANCE OF INTERTROCHANTERIC CANCELLOUS BONE QUALITY

The local intertrochanteric cancellous bone architecture, quantified by micro-CT and its mechanical properties, turned out not to be good predictors of RSA measured migration and osseointegration. The observation suggests that the significance of cancellous bone quality for the initial stability and osseointegration of cementless femoral stems has been over-emphasized. Against the hypothesis of this study, THA patients with impaired quality of intertrochanteric cancellous bone did not exhibit delayed osseointegration compared to patients with normal cancellous bone structure. Except for the micro-CT-based BMD quartiles, femoral stem migration was not dependent on any micro-CT parameter or biomechanical properties of the local cancellous bone.

Our results are against the original concept that the preservation of intertrochanteric cancellous bone during THA is important for osseointegration of cementless stems. On the other hand, our results confirm previous observations of no clear correlation between the periprosthetic bone density and the amount of bone-implant osseointegration in retrieved human ABG stems (Tonino et al., 1999). In fact, Engh *et al* (1987) had earlier reported that bone quality does not seem to have an influence on the radiographic likelihood of bone ingrowth. Despite large variability in bone quality of the cancellous bone biopsies in the current study, they still represents a rather homogenous patient group. It can be speculated that including patients less than 50 years of age (or male patients) to increase the spread of intertrochanteric bone quality would show different results. The current results demonstrate that in postmenopausal women intertrochanteric bone quality is less significant for cementless stem stability. Since our analysis demonstrated that impaired quality of the local cancellous bone is not associated with increased stem migration, the results suggest that the effect of OP on stem migration is related to other mechanisms.

Age-related changes in the cancellous bone closer to the femoral neck is different from changes seen distally along the femoral shaft. It is possible that the proximal cancellous bone biopsy not fully represent the quality of cancellous bone more distally around the femoral stem. Although the anatomic design of the ABG II stem is aimed at a proximal fixation, recent CT studies (Mueller et al., 2010) showed that common press-fit tapered-designed femoral components rely on cortical contact for stability and not on pure metaphyseal load transfer of the intertrochanteric cancellous bone region, as hypothesized by the original designers of the stems (van Rietbergen and Huiskes, 2001).

The only exception found was μCT based BMD when patients were analyzed according to BMD quartiles. Stem subsidence seemed to be inversely related to the degree of trabecular mineralization, but the effect was small. It is important to emphasize that local μCT-measured BMD is not a measure of total mass or volume of the bone, but rather a measure of volumetric density of bone minerals within the bone trabeculae, i.e. it serves as a qualitative parameter of mineralization. Increased collagen crosslinking due to accumulation of advanced glycation products and hypermineralized bone reduce the mechanical properties. Busse and co-workers (2010) reported hypermineralization of osteocyte lacunae in the proximal femur cortex of aged subjects, contributing to increased brittleness. Cancellous bone from osteoporotic and osteoarthritic patients demonstrate increased hypermineralization of osteocyte lacunae compared to age-matched controls (Carpentier et al., 2012). This could be the mechanism behind our observations, but this speculation is questionable since mechanical testing disclosed no differences in failure loads or stiffness between specimens of the BMD quartiles.

Theoretically deteriorated bone quality with accumulated bone minerals could indicate less active bone with fewer and/or less active MSCs and OBs. Impaired osteogenic conditions at the implantation

site can be compensated for by hydroxyapatite coating of implants (Soballe, 1993, Kelly et al., 2007). Despite the age-related decrease in both mechanical and microstructural properties of the intertrochanteric cancellous bone in the current study, these changes were not reflected in implant migration. It is possible that the proximal hydroxyapatite coating of the femoral stem diminished impact of the local cancellous bone quality on osseointegration.

THE SIGNIFICANCE OF AGE-RELATED GEOMETRIC CHANGES OF THE PROXIMAL FEMUR

The 2-year RSA monitoring of femoral stem migration and osseointegration included only patients with type A or B femur geometry. There were no differences in stem subsidence in patients with type A and type B femurs. Based on the anatomical stem design, high stability would be expected in patients with normal femur geometry. Supporting this hypothesis, low CFI as an indicator of changed femur geometry was identified as a predictor of delayed rotational stability. The observation indicates that indeed even minor changes of the intraosseous dimensions of the proximal femur could adversely affect the initial stability of anatomically designed femoral stems. Our result agrees with the conclusion of Noble and co-workers (1995) that cementless femoral stems of one standard shape cannot provide a close fit to the endosteal contours of elderly women. The results of the current study may not apply to femoral stems of straight non-anatomic designs relying on 3-point fixation. Attempts have been made to shape straight tapered stems for better canal fit in patients with different femoral geometry (Omlor et al., 2010), or custom-designed femoral stems (Grant et al., 2005), but standard straight double-wedged stems may work in most patients with type C femurs (Kelly et al., 2007, Meding et al., 2010, Rhyu et al., 2012). Eight patients of the original 61 recruited had Dorr type C femurs. However, these patients were previously on bisphosphonate therapy (n=3) or initiated it (n=5) after diagnosis of severe OP during screening. Stem migration in these patients did not differ from the main study patients.

THE SIGNIFICANCE OF MSC CAPACITY

RSA monitoring confirmed the hypothesis that patients with MSCs of low *in vitro* osteogenic capacity display increased stem subsidence after the settling period of 3 months, and thereby delayed osseointegration compared to patients with MSCs of high *in vitro* osteogenic capacity. Although MSCs have an essential role in cementless hip implant healing, this is difficult to prove in clinical settings due to numerous overshadowing implant and patient related factors traditionally identified to dictate successful osseointegration. By allowing measurements of micromotions not detectable with other imaging methods, RSA makes it possible to investigate impact of unexplored biological elements, including MSCs, on bone healing in cementless THA patients, reaching beyond the conventional implant related and biomechanical factors.

It is highly reasonable to believe the results truly demonstrates a relationship between MSC properties and hip implant healing independently of other factors. The two OB-capacity groups were fairly well balanced in terms of important demographic parameters (age, systemic and local BMD, rate of bone turnover, S-25(OH)D status, life style). The impact of demographic confounders known to affect not only implant osseointegration but also MSC number, quality and functions was further ruled out by correlation analyses. Although analyses of MSCs from all available patients (N=30) showed correlations between OB-capacity and clinical bone quality parameters, such correlations were not present for the 19 patients with concomitant MSC and RSA data available. Also proximal femur geometry was ruled out as a confounding factor as analysis included only patients with Dorr type A and B shaped femurs equally distributed between the two OB-capacity groups.

Still, it is possible that the decreased OB-capacity and associated delayed osseointegration is due to underlying age- and menopause-related intrinsic and extrinsic mechanisms altering the MSC capacity. Theoretically these types of underlying effects could be detected at the cellular level before manifestation in demographic or clinically measurable parameters, especially in the current study where the patients were homogenous in these respects. These alterations at the MSC-level can present as differences in osteogenic performance *in vitro* and in bone healing competence *in vivo*. Genome-wide analyses have reviled altered gene expression profiles in MSCs corresponding to ageing (Jiang et al., 2011, Alves et al., 2012), OP (Twine et al., 2014) and OA (Jiang et al., 2011), supporting a pathological role of MSCs in age-related skeletal impairment.

It could be argued that the iliac crest bone marrow MSC population not fully represents the local intramedullary cell population at the implantation site responsible for osseointegration. Iliac crest bone marrow is considered the “gold-standard” source of MSCs and should represent the general status of a patient’s MSC-capacity. Convincing evidences from comparative studies confirm this is not an issue. Studies comparing MSCs derived from femoral head trabecular bone of hip OA patients (age 57-79 yrs)(Sanchez-Guijo et al., 2009), or intramedullary MSCs from fracture patients (aged 18-72)(Cox et al., 2012) with iliac crest bone marrow derived MSCs from the same subjects have not found any differences in functional characteristics including

growth rates, surface phenotype or trilineage differentiation potentials. Further, bone grafts from iliac crest and the medullary canal obtained from fracture patients (aged 18-53 yrs) displayed similar transcriptional profile of genes related to bone repair and formation (Sagi et al., 2012). The negative effects of menopause-related estrogen deficiency on bone forming cells, especially in the trabecular bone compartment, might cause a more suboptimal osteogenic environment in the female bone surrounding the hip implant. However, since age- and estrogen deficiency-related effects on MSCs and bone formation are systemic, it is reasonable to expect same alterations in iliac crest MSCs. Bone marrow collected from reaming of the femoral canal during THA has been proven a reliable source of MSCs (Churchman et al., 2013), indicating that the rather rough treatment of the femoral canal do not negatively affect the local MSC population. Using cementless technique one can assume possible negative effects on the local cellular environment to be minimal. With cemented THA however, the thermal effect of bone cement can potentially damage the local MSCs in the surrounding tissue (Whitehouse et al., 2014).

THE CLINICAL SIGNIFICANCE

The clinical outcome of the study was good. There were no additional revisions during follow-up besides the intraoperative and early postoperative fractures (4 out of 61). The observed RSA-measured stem migration (***Study V***) and DXA-measured proximal periprosthetic bone loss (***Study III***) were not associated with any classical signs of stem loosening on radiographs during the 2-year follow-up. Despite a higher magnitude of early stem subsidence compared to previous studies, this appears to be part of the settling of the stem and seems to be within a clinically tolerated range.

The seven patients with RSA-detected unstable stems at 24 months reported mild or moderate pain but there were no radiographic indications of stem failure. One patient who reported extreme pain at 24 months was osteoporotic and had Dorr type A femur. The femoral stem migrated 2.1 mm in subsidence and 3.4 degrees in rotation during the first 6 months, but stabilized thereafter. She had however a continuous periprosthetic bone loss of 25% in zone 1, 18% in zone 6 and 42% in zone 7 at 24 months, in combination with a 14% increase in zone 3. This indicates suboptimal load transfer, which could be associated with pain. Another patient had stem rotation of 8.5 degrees within the first 6 months, and reported severe and moderate pain at the 3 and 6 months follow-up, respectively. After the stem stabilized, her subjective outcome scores increased and at 24 months she reported minimal pain, and excellent outcome scores (HHS 100, WOMAC 14). A Harris Hip Score over 80 is considered as successful outcome. In the main study population, the average HHS exceeded 80 by 6 months. The off-trial sub-population had an average HHS above 80 already at 3 months.

In ***Study VI*** the magnitude of cumulative stem subsidence from 3 to 24 months was small both in the low and high OB-capacity patients (0.7 mm and 0.3 mm, respectively), ranging up to 1 mm in individual patients, despite the statistical difference between the two groups. This magnitude of stem subsidence was not detectable on radiographs or associated with radiographic signs of loosening, and the functional recovery of the patients was uneventful. What is the impact of implant movement at this magnitude? To put it in perspective, the trabecular thickness of the intertrochanteric cancellous bone ranged between 0.14 and 0.21 mm in this particular patient group (***Study IV***). Thus, theoretically the observed migration could have impact on bone tissue that has grown into the implant surface. As demonstrated by Jasty and co-workers (1991), failure of fixation in femoral stems can occur as a result of fatigue fractures of the bridging trabeculae. However, the low systemic BMD and decreased OB-capacity of MSCs seen in our patients seem to be associated with subclinical stem migration and delayed stabilization, resembling the clinical event of slow but occurring fracture healing in OP. This does not diminish the scientific value of the current results. On the contrary, the study confirms the biological link of osseointegration with systemic bone status and MSC capacity. The results also demonstrates the sensitivity of the RSA-method to detect subclinical differences in the osseointegration process. The long-term clinical significance of the increased proximal bone loss, early stem migration and delayed osseointegration in patients with low preoperative systemic BMD or low OB-capacity of their MSCs has to be evaluated.

8.2 DISCUSSION ON THE HIP IMPLANT (ABGII)

The 2-year clinical outcome presented in this thesis study is similar to other studies using the ABG II prosthesis and other cementless HA-coated THAs.

The Anatomic Benoist Girard (ABG) II prosthesis is a second generation anatomically shaped stem. The ABG stem was developed to accommodate the natural geometry of the proximal femur, with proximal HA-coating to promote direct postoperative stability, proximal osseointegration and load transfer (van Rietbergen and Huiskes, 2001). Despite satisfactory 10-year clinical and radiological outcomes (Tonino and Rahmy, 2000) the ABG I stem was associated with high polyethylene wear and osteolysis (Gallo et al., 2010) and increased periprosthetic bone loss due to stress shielding (Tonino and Rahmy, 2000). The ABG II stem was developed aiming at improving contact between the femoral component and the surrounding bone of the proximal metaphyseal femur while preventing distal fixation.

The ABG II stem differs significantly from the ABG I stem regarding material composition, geometry, macrotexture, size and surface. The stiffness was decreased by changing the material to the titanium alloy (TMZF, Young's Modulus 85 GPa). The shoulder of the stem has extended HA-coating and is higher to improve contact and fixation with cancellous metaphyseal bone. The ABG II is shorter with a reduced diaphyseal diameter and ultra-polished to avoid distal fixation. Prospective randomized trials demonstrated better bone preservation with the redesigned ABG II compared to ABG I (Van der Wal et al., 2006, Panisello et al., 2009a), supported by finite element simulation analyses (Gracia et al., 2010). Preservation of periprosthetic bone with ABG II was confirmed in the current study (***Study III***).

The ABG II has been extensively used in Europe and Australia (Thien et al., 2014, Epinette et al., 2013, Catanach et al., 2015). At the time of patient recruitment for the current study, ABG II was among the eight mostly used stems in Finland. During 2001-2010, a total of 2,971 ABG II prostheses were implanted in Finland according to the National Institute of Health and Welfare's statistical report 2010 (www.thl.fi), representing 4.2% of all THAs (cemented and cementless). In arthroplasty register studies ABG II has shown good short-term outcome (2-2.5 years)(Mäkelä et al., 2008, Thien et al., 2014). The ABG II stem has demonstrated good clinical and radiological long-term outcome (Aguilar Ezquerra et al., 2016), with 99% survival at 8 years (Nourissat et al., 2013) and 98% by 11 years (Herrera et al., 2013). In a study of 1148 ABG II implants in French patients (age 65, 22-80 years at time of surgery) the survival rate at 14 years was 99.7% with aseptic loosening or pain as endpoints. There were 21 revisions due to fractures (Epinette et al., 2013).

Periprosthetic fractures have emerged as a leading complication in cementless THA (Hailer et al., 2010). In primary THAs, intraoperative periprosthetic fractures occur most frequently during forceful impaction of femoral stems. Tapered shape of press-fit cementless stems, female gender and increased age, the latter two probably confounded by OP, have been suggested independent risk factors of intraoperative fractures (Lindahl, 2007, Davidson et al., 2008). Despite good medium- and long-term results, the increased fracture risk associated with the ABG II stem, especially intraoperatively and in the early post-operative period is globally recognized (Mäkelä et al., 2008, Thien et al., 2014). Among 500 Australian patients with ABG II followed for 1.2-13.8 years, the total number of revisions was 17 (3.4%), of which 13 (76%) were due to periprosthetic fractures (Catanach et al., 2015). A similar pattern of increased early and late periprosthetic fractures was recently reported for a cementless femoral stem with a uniquely design resembling the ABG II (Watts et al., 2015). The high frequency of periprosthetic fractures and malpositioning with the ABG II seem to be related to the shape of the stem. In line with national and international observations, four of our 61 patients (6.6%) had a periprosthetic fracture. This appeared to be independent of the patient's BMD since the fracture cases were evenly distributed between the systemic BMD groups, supporting the theory that this is an implant-related issue.

8.3 DISCUSSION ON METHODS

The main parameters in the studies presented in this thesis were RSA-measured femoral stem migration and in vitro analyses of MSCs. Facilities, equipment and protocols for RSA and MSC analyses were meticulously set up and evaluated for the purpose of the current study.

Radiostereometry

RSA is a unique tool for monitoring implant migration, but the method is criticized for being technically demanding, expensive and time-consuming. For reliable outcome, all aspect have to be followed in great detail and analyses need to be performed by trained personnel. The set up system including all required equipment and software is expensive. Before application in patients, the system needs to be validated. Prior to the current clinical study, the applied system was standardized using a phantom model (Mäkinen et al., 2004) and the CE-certified implant was custom-modified together with the manufacturer in order to assure proper marker configuration. RSA imaging and analysis were performed by trained technicians and an experienced bioengineer, respectively. Data loss due to technical errors, loss of bone markers, invisible implant markers or other technical difficulties are common in RSA studies. In the current study, no RSA data was lost due to technical reasons, but data from one patient in the main study and two in the off-trial group were lost due to absence of bone markers.

The precision values were not as good as previously reported for the same UMRSA system from other labs. The short ABGII stem turned out to be demanding for RSA measurements. However, the precision values (0.17 mm for Y translation and 1.20 degrees for Y rotation) were closer to previous studies when the femoral head was used as an additional marker. The detection limit (limit of significance) has been applied to identify individual femoral stems displaying significant migration. For cementless stems, this is usually reported as number of stems that has exceeded the detection limit by the end of follow-up. Based on dichotomy in migration patterns of cemented cups (Aspenberg et al., 2008), we found it relevant to evaluate patients exceeding the detection limit at each follow-up time-point. This was applied to identify the time point at which each patient was found to have a stable (osseointegrated) stem. These patients are easily missed when only looking at values of migration. Comparing osseointegration time-points rather than simply comparing group means was also useful in evaluating the impact of systemic BMD and MSC-capacity on osseointegration. Applying time points of stability rather than plain numeric values of migration utilizes another dimension of RSA. In addition, the issue of data outliers is overcome. Since all stems migrate to some extent during the first 3 months, a migration largely dependent on implant design and initial stability achieved at time of surgery, the 3-24 months migration was chosen for investigating the impact of MSCs capacity, reflecting the period of biological fixation.

Micro-CT

Micro-CT has become the standard tool to quantify cancellous bone morphology and microstructure (Feldkamp et al., 1989, Burghardt et al., 2011, Boerckel et al., 2014). The good correlation between biomechanical compression properties and structural micro-CT-measured properties confirm the soundness of the method. The micro-CT method has limitations in evaluation of material properties of bone trabeculae (Fajardo et al., 2009) and it is possible that the major differences observed in the tissue density of intertrochanteric cancellous bone, i.e. in the volumetric density of bone minerals within bone trabeculae, produced artifacts in conversion of attenuation values of micro-CT imaging into equivalent tissue densities.

MSC analyses

Methods applied for bone marrow harvest, isolation, expansion and functional characterization of MSCs were modified from standard protocols at the time. Due to the high number of bone marrow samples in combination with the pace of recruitment (two/week), the study design for culture expansion and characterization assays was planned according to laboratory and incubator space as well as feasibility in terms of labor. The study protocol was strictly followed in order to obtain comparable results from all patients recruited.

SEEDING DENSITY FOR MSC ISOLATION

The increased MSC yield with low MNC seeding density in the isolation culturing is in agreement with earlier reports (Sekiya et al., 2002, Sotiropoulou et al., 2006). By reducing the seeding density of MNCs with 50%, the number of MSCs obtained was more than doubled. Several contributing factors favor MSC yield at low density. The non-MSC fraction have limited survival in culture but produce soluble factors affecting the MSCs. Diluting the MNCs further restrict survival and function of contaminating cells. Moreover, one of the hallmark characteristics of MSCs is the ability to generate colonies at low density. Applying this protocol was crucial for obtaining sufficient MSCs for characterization assays, since starting material constituted only 3-5 ml bone marrow.

IN VITRO OSTEOGENIC DIFFERENTIATION ASSAY

When initiating the project, there was no consistency in the literature regarding supplementation with

dexamethasone. As recently reviewed (Seong et al., 2010), 10 and 100 nM concentrations are used at similar frequency. The tedious task of optimizing the conditions to meet the requirements of the main project was taken on. Results are presented in ***Study II***, and the developed protocol was applied in ***Study VI*** and has been applied in subsequent studies from our lab (Alm et al., 2010, Laine et al., 2012, Heino et al., 2012, Joensuu et al., 2015).

Lack of mineralization for part of the MSCs despite repeated assays rule out methodological issues. The ratio of organic and inorganic phosphate is critical for mineralization to take place. Mineralization is largely determined by the level of ALP activity (Allori et al., 2008b). Correlation between ALP and von Kossa staining indicated reliable readout methods. The von Kossa method was applied since that was the established method in our lab at the time.

QUANTIFICATION OF ALP AND VON KOSSA STAINED CULTURES

For quantification of osteogenic differentiation and mineralization, image analyses principles were adopted from histomorphometric methods used in our lab (Valimäki et al., 2006). The method is based on computer-aided digital image analyses with a custom-designed software application (LabView graphical programming environment, National Instruments, US). Much attention was paid on developing the scanning protocol and image handling to minimize artefacts and to produce comparable images in an automated way. The method was further validated for sensitivity through titration experiments. Being able to quantify osteogenic differentiation and mineralization from fixed and stained cultures solved practical issues of conducting characterization analyses on a restricted number of MSC from a high number of donors.

Data analyses

Converting continuous variables into categorical is a reoccurring strategy in the current project. This increased the statistical power by overcoming large variability in measured values, and provided a way to focus on clinically relevant trends instead of numerical associations. Importantly, conversions into categories were performed using objective approaches, utilizing established cut-off levels (such as systemic BMD, vitamin D, uncoupling index), or technically defined cut-off levels (osseointegration). In other cases, conversion was based on quartiles.

HIGH AND LOW OSTEOGENIC CAPACITY OF MSCs

Expression of ALP represents osteogenic differentiation while mineralization represents functional osteoblasts. Since both properties are essential in osseointegration, we divided the patients into low and high OB-capacity groups based on the combined ALP and mineralization outcomes for analyzing the relationship between *in vitro* osteogenic capacity of MSCs and implant migration. The ranking strategy of indexing differentiation and mineralization properties separately, followed by calculation of a combined index represents a fairly objective way of dividing the groups based on *in vitro* MSC properties. The approach to include only patients with a combined index of 5 or 6 to the high capacity group ensured that patients with the lowest ranking index in either of the parameters (differentiation or mineralization) were designated as low OB-capacity regardless of ranking in the other parameter. Although this produced unequal group sizes, it should impartially reflect a general level of MSCs' osteogenic capacity within this study population.

8.4 LIMITATIONS AND STRENGTHS OF THE STUDY

This study carries both strengths and limitations. The high number of methods applied brings strength to the study, but the study-design carried several logistic and practical challenges, which were all solved in a solid way. From the cell culture perspective the study was challenging due to the number of samples along with the pace of recruitment (two samples a week). The amount of work required for expansion and characterization of MSCs is easily underestimated. In this case, the small starting material (3-5 ml BM) in combination with the high number of cells required for proper osteogenic differentiation experiments placed high demands on the work load and incubator space in the cell culture lab.

The study did not include gene expression analysis of the MSCs. In ***Study II***, analysis of upstream osteogenic genes RUNX2 and OSX could have provided molecular explanations to the long-term stimulatory effect of transient Dex treatment, while analysis of matrix components could have provided additional information on effects of transient and constant Dex treatment on COL1 and OCN. In investigating MSC capacity in relation to osseointegration in ***Study VI***, gene expression analyses could have exposed differences in expression patterns of RUNX2 and OSX, intermediate (ALP, COL1, osteopontin) and downstream (osteonectin) osteogenic genes, or even frequencies of committed MSCs from different patients. Such analyses could also contribute to understanding underlying molecular mechanisms to the observed low and high *in vitro* osteogenic capacities. Concomitant analyses of adipogenic genes could further explain differences in osteogenic capacity of the MSCs from different patients. While these analyses can provide information on different gene expression levels it does not always correspond to the actual protein level, and therefore not reflecting the functionality of the cells. Due to the restricted number of cells and the high work load in ***Study VI*** we were forced to prioritize readout methods. The primary aim was to analyze the functional osteogenic capacity.

By dividing patients into low and high OB-capacity groups (***Study VI***) the limited statistical power due to large variability in the MSC data was evaded. Increasing the number of patients could have increased the statistical power of our study and allowed for other statistical approaches, but to fully overcome the large variations the number would need to be unrealistically high for this type of prospective study. The rate of drop-outs was low during the follow-up, but the power of our study was limited due to low number of patients with concomitant successful MSC analysis and RSA follow-up. This was not surprising taking into account that both methods are demanding and the current study represents to our knowledge the first of its kind. With its high accuracy and precision, RSA allows minimizing the sample size. The size of the recruited cohort (N=43) and the number of patients with concomitant MSC analysis and completed two-year follow-up (N=19) were within the recommended minimum group size of 15-25 patients in RSA studies (Valstar et al., 2005), but the MSC capacity subgroups were undersized.

Although most patients were postmenopausal, measurements of serum estrogen levels could have been of value for the analysis, as time since menopause varied among the patients. Individual variation in endogenous levels of other known bone affecting agent, such as sclerostin, BMPs and TGF-β1 can also account for differences in MSC capacity *in vitro* and bone healing *in vivo*.

The study did not delineate the possible impact of gender, type of cementless stem or cement fixation on periprosthetic bone remodeling, implant migration and osseointegration. The choice of implant is both a limitation and a strength. The results may not be applicable to femoral stems of other designs or to cemented fixations. Since the ABG II is not previously analyzed with RSA, the magnitude or pattern of migration in younger patients and patients with higher bone quality is not known. It appears however as this femoral stem is associated with increased early migration, which make it possible to study and detect biological factors affecting osseointegration.

The thesis work does not show how the parameters are interconnected, i.e. the hierarchical readout when all parameters are taken into account, defying which parameters are the more decisive. The high number of parameters in combination with the restricted number of observations makes the data unsuitable for multivariate analyses. In order to do a fully reliable multivariate analysis the number of patients with concomitant data from all parameters would have needed to be larger.

One main parameter that is not part of this thesis is physical activity and muscle strength of the patients. These parameters are to be considered as major effectors on cementless THA, since especially the initial migration of the stem is probably affected by the level and frequency of physical activity. In addition, physical activity stimulate MSC proliferation and osteogenic differentiation (Luu et al., 2009). Hence the impact of the pre and postoperative physical activity on bone quality and osteogenic properties of MSCs on one hand, and stem migration and osseointegration on the other hand are not known.

A number of radiographic parameters are traditionally applied for postoperative evaluation of THA surgery, including the fit and fill of the implant in the femoral canal, varus/valgus positioning, leg

length restoration, and femoral offset. Since these parameters are familiar to the orthopedic community, reporting of these parameters in this thesis could have been beneficial for the general interpretation. Apart from surgical technique, the femoral canal fit and fill depends on the stem design and the appropriate sizing, and will affect the immediate stem stability. Applying the fit and fill analysis previously presented by Mont and his team (Issa et al., 2014) could have provided useful information on the possible impact of stem sizing on early migration.

In order to demonstrate the impact of bone quality and the role of MSCs in the progress of cementless implant healing in clinical studies, the key is to minimize the many possible confounding factors by applying strict exclusion criteria, yet to select patients with increased potential of exhibiting a certain degree of implant micromigration before eventual osseointegration. For example, in younger (less than 65-years old) male and female patient populations with good skeletal health RSA-migration is minimal or not even detectable. Postmenopausal aging women represent this type of an ideal study population not only for the scientific purpose but for the clinical relevance: First, postmenopausal women represent the largest group of patients undergoing THA (Mäkelä et al., 2010, Sadoghi et al., 2012). Second, they are prone to impaired skeletal quality (Glowacki et al., 2003) with increased risk of complications including delayed osseointegration (Vandamme et al., 2011) and periprosthetic fractures (Sidler-Maier and Waddell, 2015). Third, they display an age-related decrease of MSC capacity (Zhou et al., 2008).

The strength of the study is characterized by several factors. The homogenous and carefully screened female population brought strength to our study. The well-defined exclusion criteria ensured inclusion of only female patients who suffered from primary hip OA but were otherwise healthy and without bone-affecting medication. The study benefited from DXA measurements of numerous anatomical sites, including both weight-bearing and non-weight-bearing long bones, aside with collaborating biochemical evaluation of bone turnover. All patients underwent standardized surgical procedure performed by a single experienced orthopedic surgeon, receiving the ABG II anatomically-shaped femoral stem with expected minor stress-shielding effects on the proximal femur, size-fitted to obtain optimal press-fitting, limiting possible confounding factors. The use of ceramic-ceramic bearing surfaces was aimed to minimize generation of wear-particles as a potential further confounder.

The combination of methods applied in the current thesis represent a unique design for studying biological parameters affecting osseointegration of a cementless femoral stem in postmenopausal women. The applied methods allowed for investigating the impact of both systemic and local bone biology factors at the blood chemistry, skeletal (DXA), tissue (bone biopsy, μCT, biomechanical testing) and cellular (MSCs) level on bone remodeling (DXA) and implant migration and osseointegration (RSA). Application of the sophisticated RSA method for longitudinal monitoring of implant micromigration, in combination with analysis of individual patients' MSCs makes the study unique. One of the shortcoming was the low precision of the applied RSA compared to studies in the literature. Next step in the investigation of osseointegration and MSCs in postmenopausal women undergoing cementless THA include physical activity, muscle strength and migration of the acetabular component.

8.5 GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Although million THAs are performed annually, little is known about factors actually affecting osseointegration. Osteoporosis, osteopenia and aging are considered contraindications for cementless THA. Imbalanced remodeling and increased fragility of bone associated with these conditions are suggested risk factors for impaired osseointegration and decreased long-term implant survival. This is further supported by documented decrease in number and osteogenic capacity of MSCs with aging, dictating the bone healing potential. In reality however, understanding of fracture healing and osseointegration under osteoporotic conditions is still limited. What evidences are there of increased failure of cementless femoral stems in OP patients, and what is the impact of OP and osteopenia on osseointegration and long-term outcome of cementless THA? Is it really a question of bone quality, or just a question of time?

Initial stability achieved at time of surgery is a prerequisite for biological fixation to take place, hence affecting the long-term survival of cementless femoral stems. The impact of implant-related factors for obtaining initial stem stability is undisputed, as evident from trials and errors throughout the 40 years of cementless THA of numerous stem designs. With modern designs, previous complications have been overcome and cementless femoral fixation has excellent long-term outcome with many implant types presenting >95% 10-years survivorship and persisted 20-year survival >90% with some stem designs (Khanuja et al., 2011, Pivec et al., 2012). This has resulted in two things; 1) indications for cementless THA has extended and is now used in broader spectrum of patients, including aged prone to low systemic BMD, and 2) the impact of patient related factors and other non-implant factors become more evident. It is realistic to consider compromised bone quality to interfere with the normal course of osseointegration, especially in severe OP and very old (female) patients, contradicting the use of cementless THA in these patients. However, the current study show that:

- The typical patient group receiving cementless THA has poorer systemic bone quality than expected
- Low systemic BMD, even at a subclinical level (osteopenia), has major impact on early stem migration and osseointegration
- *In vitro* assayed level of osteogenic potential of the patients' MSC do correlate with stem migration and osseointegration
- Eventually most implants stabilizes

This encourages further research to better understand the biology of osseointegration in general, and in aging and OP in particular. Traditionally, implant-related factors have dominated the research focus, along with demographic factors. Although MSCs are not the entire story in osseointegration, they are key cells responsible for bone formation and have a role in age-related skeletal deterioration (**Figure 8.1**). With the increasing need for artificial joints that can be functional and withstand an active lifestyle for 20-30 years, new implant designs may not be the only approach. Better understanding of the role of endogenous MSCs can be an additional tool and a target for improving future outcome of THA.

OSSEOINTEGRATION OF CEMENTLESS FEMORAL STEMS – A SYSTEMIC PROCESS?

Principles of the osseointegration process are known in theory but are not well understood in human patients. Results presented in this thesis supports the idea of osseointegration of cementless stems as a systemic process.

Hip replacement is a major skeletal intervention, triggering repair and biological responses at the systemic level, detectable as changes in inflammatory, fibrinolytic, and coagulatory cascades (Clementsen et al., 2006) and bone metabolic markers (Kenanidis et al., 2010) in blood following THA. The biological processes taking place, including periprosthetic bone healing in response to the surgical trauma, bony ingrowth/ongrowth, and adaptive remodeling of the proximal femur, engage all cellular and molecular parts of the bone remodeling machinery. OP and aging are generalized systemic conditions affecting cells, metabolism, remodeling and quality throughout the skeleton (Rachner et al., 2011). Further supporting the idea of THA-healing as a systemically influenced process are the numerous factors known to affect the healing process including medications affecting bone metabolism, non-steroidal anti-inflammatory drug (NSAID), smoking and alcohol consumption, physical activity and muscle strength. Therefore, it is not surprising that systemic BMD status (***Study V***) and MSC capacity (***Study VI***) were found as significant predictors of stem migration and osseointegration along with aging (***Study V***) in the postmenopausal women of the current study, while quality of local intertrochanteric cancellous bone had less impact (***Study IV***). Factors affecting osseointegration and their possible interactions are summarized in **Figure 8.1**. The current study has established a foundational base for future studies applying RSA for investigating biological factor in osseointegration.

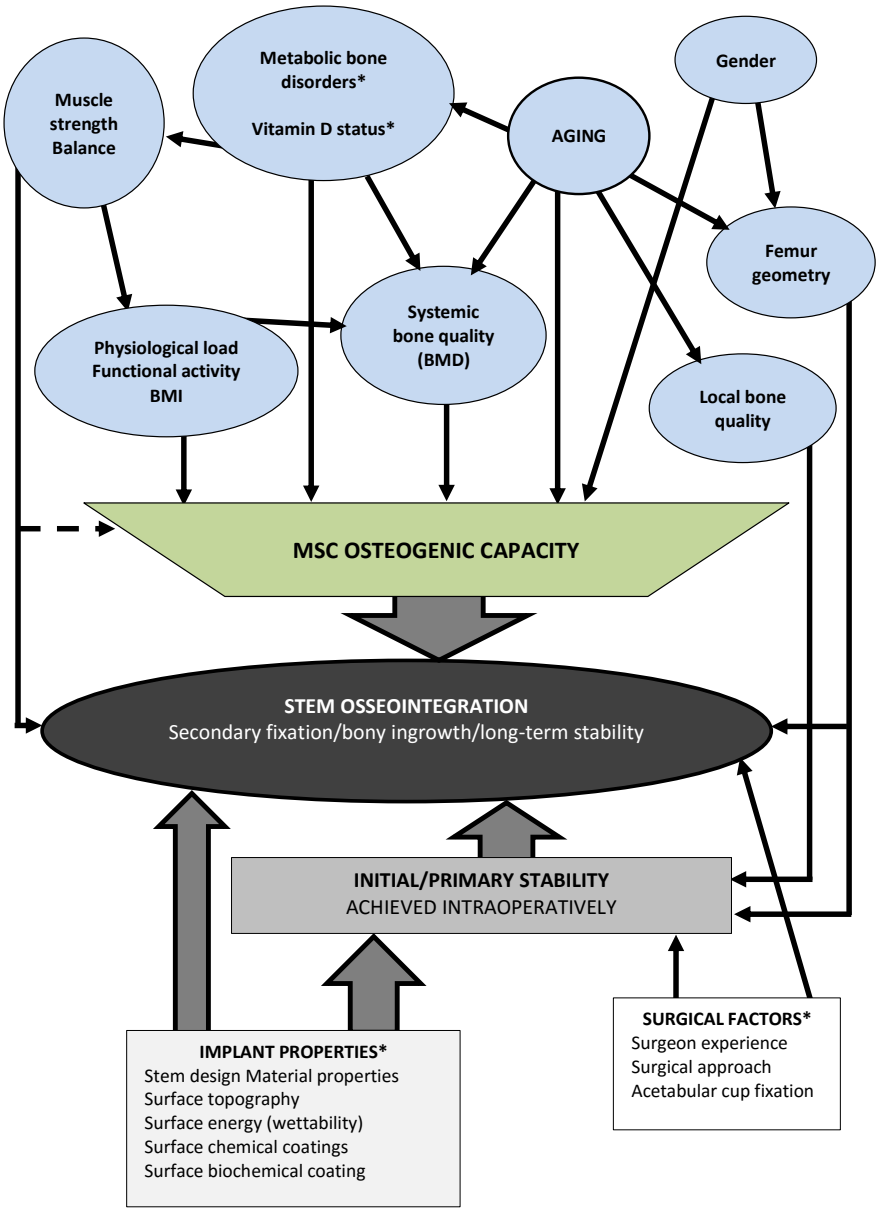

FIGURE 8.1 Overview of factors influencing osseointegration of cementless femoral stem and their potential interactions. *In addition to well investigated traditional implant-related factors (lower part of figure) a number of patient-related factors (upper part) are generally recognized as potential determinants, but the impacts of these factors are not well understood. Although MSCs are not the entire story in osseointegration, they represent the key cell type responsible for proper bone formation and healing, and are known to be affected by a range of demographic factors. Initial contact and minimal relative motion are prerequisite for the biological processes to take place, whereas surface properties of the implant and quality of the host bone contribute to cellular responses and secondary fixation, ultimately determine the overall success or failure of the implant. Asterisks indicate factors that can be controlled for.*

OSSEOINTEGRATION AND OSTEOPOROSIS – IS THERE A CONTRADICTION?

Increased fracture risk is the main clinical consequence of OP, which is a significant concern for both the individual patient and for society (Armas and Recker, 2012). The impact of OP on osseointegration is not known, but is considered as a risk factor in cementless THA due to increased fragility and supposedly decreased healing capacity. The generalized view of impaired bone healing in OP is largely based on preclinical studies, while clinical evidence is limited. Experimental animal models of OP have demonstrated delayed and altered fracture healing (Egermann et al., 2008) and osseointegration (Chatterjee et al., 2015). In patients however, fractures heal in a fairly predictable manner despite osteoporotic conditions. Nikolaou and co-workers (2009) confirmed a delayed fracture healing in osteoporotic patients compared to controls, yet demonstrating that eventually all fractures healed. Similarly, osseointegration in the current study was delayed in patients with low systemic BMD, but the majority had stabilized by 24 months (***Study V***). Thus, the increased subsidence and delayed osseointegration observed in these patients was subclinical, indicating there is reason to monitor this type of patients more closely during the first 1-2 years after cementless THA. This however requires preoperative identification of patients with low systemic BMD at risk of increased stem migration and/or delayed osseointegration.

Interestingly, previous clinical studies have indicated that neither OP *per se* (van Wunnik et al., 2011) nor quality of the bone matrix (Heetveld et al., 2005) are risk factors for disturbed fracture healing. Supporting the findings in the current study, age-related decrease in bone healing capacity has been suggested to be more important than the quality of the local bone tissue in determining bone repair in elderly patients (van Wunnik et al., 2011, Feron and Mauprivez, 2016). Similar to fracture healing, recruitment of MSCs and OBs are crucial for osseointegration. The difference in the two healing processes seem to be the source and recruitment route of contributing cells (Alm et al., 2010). Though the age-related decline in MSC number and function reduce the healing capacity and delay repair, it is encouraging that also very old (80-95 years) female hip fracture patients have functional MSCs (Alm et al., 2010). Biologically, the impact of OP and osteopenia on the bone healing process itself seem minimal.

CEMENTLESS STEMS IN POSTMENOPAUSAL AND ELDERLY WOMEN WITH DECREASED BONE QUALITY AND OSTEOGENIC CAPACITY OF THEIR MSCs

These is no consensus on the best fixation for THA in general, and the subject is even more controversial when it comes to aging and osteoporotic patients. What are the benefits of using cementless femoral stems in postmenopausal and aging women prone to OP and osteopenia, and with decreased MSC function?

Opposite to previous recommendations, increasing number of studies report successful outcome of cementless THA in patients of increasing age, with OP and with Dorr type C bone (**Table 2.3**). The global trend show increased use of cementless solutions also in older patients (Troelsen et al., 2013, Sadoghi et al., 2012, Mäkelä et al., 2014). Conclusions from these and other register studies are conflicting regarding favorable outcome of one fixation method over the other. Regardless of the reported message, results demonstrate >95% 5 year survival, and >90% 10 year survival of cementless femoral stems, with better or equal survival rates of cemented and cementless THA in patients over 55 years, with similar survival in patients over 75 years (Mäkelä et al., 2010, Wechter et al., 2013, Wyatt et al., 2014). RSA monitoring of cementless stems in aged patients with femoral neck fractures indicated minimal stem subsidence at 2-years (Skoldenberg et al., 2011b, Figved et al., 2012), with good stability but high periprosthetic bone loss and late-occurring fractures (Skoldenberg et al., 2014). Schewelov and co-workers (2012) reported that despite major early migration in hip fracture patients aged 70-96 years, stems stabilized by 2 years with good clinical outcome. A Swedish register study suggested lower risk of revision with cementless compared to cemented stems (Hailer et al., 2010). Taken together, reports in the literature indicate; 1) good long-term outcome of cementless THA, 2) cementless THA is safe to use also in older patients, and 3) the disadvantage with cementless THA is the increased risk of periprosthetic fracture, especially during the early postoperative period (**Box 10**). Despite increased use of cementless THA in an aging population, with postmenopausal women at risk of undiagnosed low systemic BMD representing the largest patients group, there are so far no randomized clinical trials focusing on elucidating the effect of OP and osteopenia on osseointegration.

It can be argued that cemented fixation is a more attractive alternative in patients at risk of decreased MSC capacity and decreased bone health. However, MSCs may also have a role in the long-term success of cemented prostheses. Cement fixation relies on mechanical interlocking in cancellous bone regions, but failed osteogenic function of MSCs may impair bone remodeling, leading to structural weakness of periprosthetic cancellous bone and subsequent mechanical loosening of a cemented prosthesis. Autopsy studies have demonstrated micromotions at

the bone-cement interface which increase over time because of morphological changes due to remodeling (Cherukuri et al., 2010). Further detailed analysis of radiologically fixed stems have reported significantly higher bone-implant contact and less interface micromotion with cementless compared to cemented stems (Mann et al., 2012). The osteogenic capacity of MSCs may be important also for the long-term survival of cemented hip prostheses, although this is still an unexplored field of research.

There are several reasons why not to use cemented fixation. Intra-operative complications related to cementing include sudden death and increased fatal cardio-pulmonary complications. Mortality is related to marrow, fat and bone embolism, along with release of cement particles to the bloodstream and lungs with increased risk of mortality (Parvizi et al., 1999, Issack et al., 2009, de Froidmont et al., 2014). The longer surgery time compared to cementless procedures increases the risks in elderly patients. Cemented THA is associated with increased intraoperative, early postoperative and long-term mortality (McMinn et al., 2012). Cementing is also associated with increased occupational hazard to the surgical staff. Long-term aspects include an increased risk of aseptic loosening and infections, but also dislocations and fractures (Mellon et al., 2013). With increasing age, the risk of complication increases, and revision after cemented THA can potentially be more demanding due to loss of bone quality and mass. Clinical reports indicate that OP is a risk factor for periprosthetic fractures and late mechanical loosening of cemented hip prostheses due to poor mechanical and biological properties of bone (Broden et al., 2015). Necrosis of bone cells and tissue due to cementing further deteriorate the weak bone stock over time (Whitehouse et al., 2014), which can have disastrous long-term effects and complicate revision surgery (Sheth et al., 2015).

BOX 10. Current benefits and disadvantages of cementless THA in aging patients
STRENGTHS AND BENEFITS
Lower short- and long-term mortality
Shorter surgery time
Easier surgical procedure
Greater choice of bearing surface
Ability to remodel and repair periprosthetic bone
Allow bone to adapt to changes in load transfer
Fewer complications
Minimal risk of aseptic loosening
Revision easier
Proven safe in aged and osteoporotic patients
Documented good long-term outcome records
RISKS AND CHALLENGES
Press-fitting challenging in osteoporotic bone
Achievement of initial stability
Increased intra- and postoperative fracture risk
Delayed osseointegration due to:
Lack of initial stability
Decreased osteogenic potential
Late loosening due to fragile bone ingrowth
Mellon et al., 2013, McMinn et al., 2012, Carli and Jerabek 2015

CEMENTLESS THA IN AGED AND OSTEOPOROTIC PATIENTS – CONCIDERATIONS AND SOLUTIONS

What are the considerations when using cementless THA in aging and osteoporotic patients? Do the benefits outdo the increased fracture risk, and can fractures be prevented?

Press-fitting a femoral stem into an osteoporotic femur can be difficult and there is a worry whether the weakened femoral bone is strong enough for the insertion. Next concern is whether the initial stability can be maintained once it is successfully inserted. There is an increased risk of both intraoperative and postoperative fractures. Potential long-term complications include loss of optimal implant position, delayed osseointegration due to decreased osteogenic potential, and late loosening due to mechanical failure of the ingrown trabecular bone. Since the process is slower, achievement of initial implant stability is even more important. But if the implant is stable enough bony fixation will take place similarly as in patients with normal systemic BMD and eventually also osteoporotic bone heals. Thereafter the challenge is to maintain a proper fixation and prevent fractures.

If cementless THA is used in aging and/or osteoporotic patients, good systemic bone quality needs to be cared for. Considering current estimates, there will be an increasing number of aging patients with cementless THAs in the future. Results presented in this thesis suggest that (female) patients scheduled for cementless THA should be more carefully screened for systemic BMD status (DXA), secondary causes of OP (laboratory tests), and geometric femoral changes of the affected hip in order to identify patients at risk of delayed implant healing. The significance of periprosthetic bone loss is still unclear and evidences for clinical consequences are scarce. Bisphosphonate therapy has indicated short-term prevention of periprosthetic bone loss (Skoldenberg et al., 2011a), but there are no evidences of beneficial effects on survival of cementless femoral stems.

Developed to improve osseointegration (Furlong and Osborn, 1991), hydroxyapatite coating has excellent outcome records over the last 30 years, and many cementless femoral stems are manufactured with HA coating (Herrera et al., 2015), presenting favorable outcome compared to non-coated stems (Epinette and Manley, 2008). However, HA coating has been questioned based on clinical

(Flatoy et al., 2016, Camazzola et al., 2009) and register studies reporting no additional clinical benefit. This is a concern, since these studies are not always optimally designed to investigate the benefit from HA in aged and osteoporotic patients. In the Swedish (Lazarinis et al., 2011) and the Nordic (Hailer et al., 2015) arthroplasty registers both HA-coated and uncoated cementless stems have 98-99% 10-year survival. With this high success rate, it is difficult to prove additional beneficial effects of HA. Randomized clinical trials involve mainly younger patients with good bone and healing capacity, not necessitating HA-coating for successful fixation. More focused studies however demonstrate that HA can improve both the quality and the rate of fixation (Luites et al., 2006), even in osteoporotic bone (Skoldenberg et al., 2011b), in revision (Salemyr et al., 2008) and in aged patients and patients with Dorr type C femurs (Kirsh et al., 2001, Kelly et al., 2007). These reports encourages the use of HA-coated stems in patients with compromised bone.

Documentation of cellular and molecular changes in the bone forming machinery with aging and menopause, along with increasing understanding of the different wnt signaling pathways and their regulations, new targets for drug development are revealed (Lerner and Ohlsson, 2015), switching focus from antiresorptive to formation promoting strategies. These strategies are promising for future applications in cementless THA to promote proper osseointegration, especially in aged and osteoporotic patients. An increasing awareness among health professionals and patients regarding bone health can contribute to a generally improved and maintained bone quality in the aging population.

APPLICATION OF RSA FOR MONITORING OSSEOINTEGRATION OF CEMENTLESS FEMORAL STEMS

Radiostereometric analysis allows for precise quantification of implant migration. It is a unique method for monitoring any type of implant migration at the sub mm level. What is the significance of RSA application for clinical practice, and what is the predictive value of RSA-measured migration of cementless femoral stems?

Application of RSA has developed over the last 35 years, with an increasing number of established RSA research groups around the world. Although the original application of RSA was to improve early detection of implant failure (Baldursson et al., 1979), the value in arthroplasty research today is in evaluating new implant designs and for studying the biology of implant healing, which is much in line with the original idea of RSA for studying skeletal biology and development (Aronson et al., 1977). Two distinct features of cementless femoral stem osseointegration have been explained through RSA studies. First, the main migration seem to take place within the first 3-6 months when the stem settles, regardless of stem design (**Table 2.3**). This can be regarded as part of normal healing and adaption to the altered mechanical situation following implant surgery. Second, continuous migration after 3 months or beyond can indicate slow or unsuccessful osseointegration. With model-based RSA techniques (Kaptein et al., 2003) the expensive process of regulatory approval and manufacturing of modified implants for attaching tantalum markers has been overcome, making RSA screening of new implants more available.

RSA is not intended as a routine assessment for clinical follow-up. As for now, assessment of THA outcome in clinical practice relies on visual interpretation of conventional radiographs for identification of radiographic features and the semi-quantitative radiological scoring according to Engh *et al.* (1990). The relationship between plain radiographic analysis and RSA-measured migration is currently unclear. EBRA offers an alternative method for measuring stem subsidence from radiographs. Since it do not require bone or implant markers, it is applicable to all patients, but accuracy is low and it does not allow for measuring rotational migration (Biedermann et al., 1999). Recently, a marker-free automated CT-based spatial analysis method was presented as an alternative (Scheerlinck et al., 2016). Offering 3D migration analysis from CT images with accuracy and precision comparable to RSA, the method is attractive since it is applicable in most patients and can be applied in clinical practice.

It is currently unclear how well RSA can predict the risk of later loosening of modern cementless implants. In a recent attempt to clarify the value of early RSA-measured migration on later implant failure, van der Voort an co-workers (2015) analyzed 24 RSA studies and confirmed an association between subsidence and late loosening for cemented stems, while this could not be detected for cementless stems. With RSA, femoral stems at high risk of failure can certainly be identified as presenting with major migration, but most implants today show only minor or moderate early migration followed by stabilization. This is confirmed by an increasing number of midterm (5-6 years) RSA follow-up reports (Ström et al., 2003, Wolf et al., 2010, Callary et al., 2012, Nysted et al., 2014, Weber et al., 2014, Nebergall et al., 2016).

Interpreting RSA data has its own pitfalls. Presenting data as mean (or median) values, according to the RSA guidelines (Valstar et al., 2005), is useful when evaluating new implant designs or investigating the impact of medical intervention on implant migration. But there is a risk that patients in

true need of monitoring are missed, and the clinical relevance of small but statistically significant differences in mean migration has been questioned (Aspenberg et al., 2008). Further, since what is actually measured is the change in stem position compared to baseline, the true distance of migration over time gets hidden when mean values are presented. Analyzing migration patterns at the individual patient level may reveal subjects with delayed stabilization and/or deviant migration patterns, as demonstrated in the current study (***Study V-VI***). Migration along the y-axis is usually only in one direction, i.e., subsidence along the femoral canal, but as observed in some patients of the current study, rotation around the y-axis can occur in anteversion and retroversion at different follow-up intervals, making the actual distance of migration over time greater than just the indicated change of stem position compared to baseline (**Figure 8.2**). This indicate distinctly different healing courses in categories of patients with different biology at the implantation site (Aspenberg et al., 2008), or in systemic bone biology (***Study III-VI***). High-resolution digital image analysis of autopsy retrieved radiologically fixed cementless stems have confirmed a significant inverse relationship between bone-implant contact and magnitude of interface micromotion (Mann et al., 2012). By applying experimentally controlled axial torsional load representative of gait and stair climbing, micromotions ranging between 0.3 and 2.6 µm were recorded, whereas motions up to 2.3 mm were recorded for radiologically loose stem. These observations support the rationale of applying RSA detected cessation of micromigration as a sign of osseointegration, and monitoring of cementless stems despite yet unidentified cut-off levels. The next step will be the determination of long-term performance of cementless stems in osteoporotic female patients. Repeated RSA studies and functional evaluation after 5 and 10 years will reveal the impact of the increased early subsidence and rotation demonstrated in the current study.

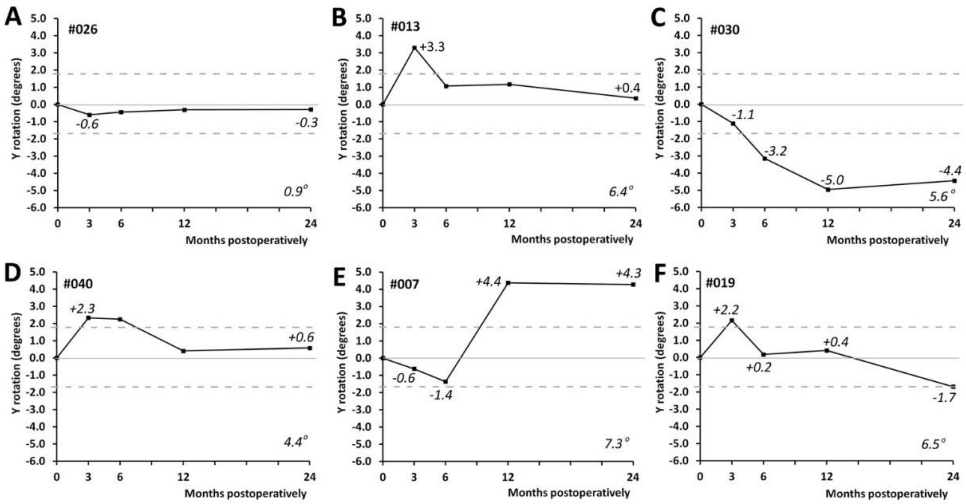

FIGURE 8.2 *Examples of different patterns of RSA measured axial rotation of cementless femoral stems from the current study. The upper row show axial rotation in three patients displaying different patterns typically reported in RSA studies of cementless stems. Patient #026 (A) represents no rotational migration, while patient #013 (B) demonstrate the typical pattern of early (3-6 months) migration followed by stabilization. Patient #030 (C) represents a case of late stabilization, with continued rotational migration up to 12 months. The lower row demonstrate three patients with deviating migration patterns that are easily missed when analyzing group means or change in stem position compared to baseline. This is particularly evident in patients #040 (D) and #019 (F). The cumulative distance of rotation for each patient is indicated in the lower right of the graphs, demonstrating the discrepancy between RSA measured change in stem position at 24 months compared to baseline and the actual distance of migration. Dotted lines=detection limits based on precision values.*

MIGRATING, STABILIZED OR OSSEOINTEGRATED?

RSA provides an indirect measurement of stem stability/fixation. What does RSA-measured stem migration or lack of migration after the initial settling period indicate? Is migration a sign of failed osseointegration, is lack of migration an indication for successful osseointegration?

A certain degree of early migration is expected with cementless femoral stems, and this non-continuous migration should not be used as an indicator of failed osseointegration. However, attention should be paid in case of continuous migration or late appearing migration of previously stable implants. Contradiction has been reported in RSA data for stable and migrating cemented cups (Aspenberg et al., 2008), but this has not been tested for cementless stems. An implant might not be osseointegrated although migration is not detected with RSA, depending on the precision of the applied RSA system. Other technical explanations include timing of baseline measurements and the stability and distribution of the bone markers, i.e., the mean error and the condition number, respectively. Another possible scenario of an undetected migrating stem is a case of an implant rotating back and forth. The other way around, an implant can be osseointegrated despite some degree of RSA-detected micromigration. It is theoretically possibile that an osseointegrated stem display some degree of migration due to high mechanical flexibility of the ingrown bone tissue, for example in case of some degree of fibrous tissue or low mineralization. This can be seen with RSA in fracture healing (Madanat et al., 2012), but has so far not been described in cementless THA.

There are four underlying explanations for late appearing or continuous migration of a cementless femoral stem; 1) it never osseointegrated due to technical failure such as malalignment or undersizing of the implant, leading to lack of fit and proper contact with the surrounding bone, 2) continuous micromotions prevent osseointegration due to unfavorable femur geometry or lack of initial stability 3) the bone is biologically not conductive enough to achieve proper fixation, and 4) the established bony anchoring breaks because of low bone quality. Simplified, scenarios 1-2 describe migration and failed osseointegration due to lack of proper bone contact, which can be improved through right surgical choices in terms of implant design and surgical techniques. In scenarios 3-4, lack of proper bone quality lead to impaired bony fixation, which should be the focus of future research to solve.

IN VITRO OSTEOGENIC DIFFERENTIATION OF MSCs - CONCIDERATIONS AND INTERPRETIONS

Cell culture represents simplified conditions and *in vitro* assayed MSC properties should always be interpreted with caution, also because MSCs undergo significant changes in culture (Qian et al., 2012, Larson et al., 2008), and cells isolated from a compromised *in vivo* environment may display increased response when subjected to optimal *in vitro* culture conditions (Zhou et al., 2012, Brunt et al., 2012, Fickert et al., 2011). Therefore, analyses of fresh rather than cultured cells has emerged as an alternative approach for characterizing MSC profiles in different musculoskeletal disorders. From freshly isolated cells phenotype and gene expression profile can be obtained, which can reveal age- and/or disease-related pattern variations. Although a highly relevant approach, detailed strategies require further development. Selecting the "correct" cell population from fresh bone marrow is one issue. Prospective isolation can be performed with flow cytometry (Qian et al., 2012), but defying the optimal cell surface marker combination(s) is a challenge. Culture-based experiments are still needed for functional assessment of MSCs, often with prior culture expansion to meet the high cell number requirements of these assays. There have been little development regarding readout methods for evaluating i*n vitro* osteogenic capacity of MSCs, and the traditional analyses are largely applied.

In relation to skeletal aging and musculoskeletal diseases, the question sustains how much culture adapted MSCs can tell regarding the bone formation capacity status of the donor or disease-related cellular alterations. There are documented correlations with certain demographic parameters, but the *in vitro* osteogenic response of human MSCs can be inversely related to demographic factors, as demonstrated with vitamin D deficiency (Zhou et al., 2008)(***Study VI***) and BMI (Friedl et al., 2009b). *In vitro* and animal studies have indicated that it is possible to "rescue" the bone forming capacity of MSCs by nutritional, pharmacological and cellular therapy (Zhou et al., 2012, Brunt et al., 2012, Baker et al., 2015), and *in vitro* conditions may facilitate such rescue actions. This can interfere with the value of *in vitro* analyses, but it also provides an approach for therapeutic targeting. The "diagnostic" value of *in vitro* osteogenic analyses of MSCs at the individual patient level is yet to be confirmed.

THE ENDOGENOUS MSC POOL IN OSSEOINTEGRATION – ROLE AND FUTURE THERAPEUTIC TARGET

Osseointegration is a complex process affected by patient-related, surgical, implant-related, biomechanical, and biological factors. Although the role of MSCs is not the entire story, it is the least explored part of the osseointegration process. Can MSCs be utilized as tools and therapeutic targets for enhancing osseointegration and bone healing in aging and disease?

Several factors are involved in regulating MSCs during osseointegration but mechanisms are poorly understood. Results presented in this thesis suggests that *in vitro* analysis of the "osteogenic fitness" of the MSC reservoir may serve as a unique clinical model to verify and evaluate the role of MSCs in the process of implant healing, particularly in patients at risk of age-related dysfunction of MSCs and decreased bone healing capacity.

So far, two research focuses have dominated the MSC field. One aiming at clarifying the impact of demographic parameters and disease conditions on MSC properties, as defined by *in vitro* performance or gene expression profiles. The other is development of therapeutic applications of MSCs for improving, correcting and/or replacing damaged and diseased tissues, or inhibiting trauma or disease-related conditions. Less focus has been on elucidating the biological role of endogenous MSCs in bone formation and repair in humans. Experimental animal studies have provided valuable insights into *in vivo* function of MSCs, and emerging clinical trials have confirmed the participation of MSCs in bone healing. Yet, the *in vivo* mechanisms and routes of MSC recruitment in bone formation and healing are to be described. Known molecular and cellular mechanism are largely based on fracture research while osseointegration, especially in long-bones, is largely undescribed. The two processes are considered partly overlapping, but there are fundamental differences. As we previously reported, MSCs are temporarily released into the circulation following fracture, but not following THA (Alm et al., 2010).

Despite great expectations, application of ex vivo expanded MSCs for bone repair has proven challenging (Dawson et al., 2014). Individualized aspects are emerging in orthopaedic medicine, where MSCs present a key tool in tailoring future improved implant osseointegration, and the results in this thesis further supports this concept. Gene expression analyses has identified subsets of osteogenic markers able to identify an osteoporotic phenotype in cultured human MSCs (Twine et al., 2014), and a gene signature for donor age (Alves et al., 2012). It is possible that corresponding subsets of gene markers in MSCs could predict the bone healing in orthopaedic patients.

A number of signaling molecules affect MSC survival, proliferation, recruitment and differentiation. Individual variation in endogenous levels of these factors can account for differences in both *in vitro* properties of MSC and *in vivo* healing responses upon THA surgery. One such factor is sclerostin, which levels in serum and bone marrow increase with aging (Roforth et al., 2014, Modder et al., 2011). Increased serum levels of BMP-2 have been found in patients with knee OA (Liu et al., 2015), while circulating level of TGF-β1 increase after fracture (Sarahrudi et al., 2011) with altered levels in delayed fracture healing (Zimmermann et al., 2005). Currently, there are no corresponding data available regarding these factors in the circulation following THA, but it is possible that individual variations are part of the underlying mechanisms contributing to the observed differences in OB-capacity and osseointegration in the current study.

Notably, the current study analyzed *in vitro* MSC properties reflecting the preoperative status. The longitudinal nature of the RSA data reflect an *in vivo* process that *per se* is much more complex, and is influenced by a number of possible changes triggered by the surgical intervention as well as life-style/patient related parameters over the 2-year follow-up period. The study provides a proof of concept, presenting a novel approach for studying the biological aspects of hip implant osseointegration and to verify the impact of decreased MSCs function. Further research is warranted for more detailed analyses of contributing molecular factors and possible mechanisms.

The implant surface properties regulate MSCs at several levels. Implant topography and coating properties can alter the gene expression, proliferative activity, secretome and differentiation of MSCs (Galli et al., 2012), mainly through activation of the non-canonical (Olivares-Navarrete et al., 2011) and the canonical wnt signaling pathway (Thorfve et al., 2014), respectively. Targeting wnt inhibitors, such as sclerostin, should therefore improve osseointegration in aging and postmenopausal women.

A number of strategies are under development focusing on boosting, mobilization and homing of endogenous MSCs for improved bone repair. The stromal cell-derived factor-1 (SDF-1) – CXCR4 axis, and VEGF represent key targets for MSC stimulation (Herrmann et al., 2015). Recruitment alone may not be enough, the conditions at the healing site also needs to be adequate. Systemic conditions including OP and advanced age can alter the optimal inflammatory conditions needed for proper healing (Einhorn, 1998). Targeting the interactions between MSCs and the immune cells is another potential therapeutic strategy for enhancing natural bone

healing and osseointegration. The detailed profile and functional characteristics of MSCs seem to be a product of their specific environment. This plasticity is a biological benefit, but a challenge in research. Although the responsiveness of MSCs to altered systemic conditions contribute to decreased bone formation and skeletal health with aging and menopause, it also means it can be utilized therapeutically to improve bone health and osseointegration in aging (and postmenopausal) patients. As demonstrated in this thesis project (***Study II and VI***) and in our previous study (Alm et al., 2010) these patients do have the required MSC reservoir - it may just need boosting for enhanced osteogenic functionality. Improving the systemic bone biochemical environment for instance by ensuring adequate vitamin D levels, possible monitoring estrogen levels, and if necessary applying bone formation stimulating drugs, should improve bone quality and healing capacity in patient groups at risk of delayed or failed osseointegration.

9 CONCLUSIONS

As a general conclusion observations made in this study suggest that decreased skeletal health, such as low systemic BMD and decreased osteogenic properties of bone marrow MSCs, has major influence on early stability and osseointegration of cementless hip prostheses in female patients. Although the long-term consequences of the observed stem migration and other clinical implications remain unknown, DXA screening for low systemic BMD could be used in postmenopausal patients before THA for identifying patients in need of prophylactic interventions. Based on the results, the following conclusions can be made:

I Against a general belief, OA does not seem to protect a patient from generalized primary OP. The majority (74%) of women with hip OA was osteopenic or osteoporotic with signs of increased bone turnover. In addition, an unexpectedly high number of them required preoperative endocrinology consultation due to abnormal laboratory findings related to calcium metabolism.

II *In vitro* osteogenic differentiation of human bone marrow MSCs can be promoted by transient supplementation with 100 nM Dex during the first week of induction culturing. With this protocol, it is possible to produce *in vitro* functional OBs from bone marrow MSCs of different donor populations in a fairly reproducible way, with reduced inter- and intraindividual variations.

III Women with low systemic BMD showed increased periprosthetic bone loss in Gruen zone 7 compared to patients with normal systemic BMD. The local preoperative BMD of the operated hip did not predict the bone loss, probably due to the erroneous increase of local BMD in the femoral necks of osteoarthritic hips. The anatomically designed femoral stem preserved a close to normal periprosthetic BMD in women with normal BMD.

IV The quality of intertrochanteric cancellous bone, quantified by micro-CT and biomechanical testing, had less influence on RSA measured migration of the anatomically designed cementless femoral stems than expected.

V Low systemic BMD, changes in intraosseous dimensions of the proximal femur and aging adversely affected initial stem stability and were associated with delayed osseointegration.

VI Postmenopausal women undergoing THA for primary hip OA show large variability in the osteogenic capacity of their bone marrow MSCs. Decreased *in vitro* osteogenic properties of MSCs was associated with increased stem subsidence after the settling period of 3 months, and thereby delayed osseointegration. The study provides a proof of concept suggesting that *in vitro* analyses of the osteogenic properties of autologous MSCs in patients scheduled for orthopaedic surgery can be of relevance.

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