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In vitro osteogenic capacity of bone marrow MSCs from postmenopausal women reflect the osseointegration of their cementless hip stems

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ABSTRACT

Age-related dysfunction of mesenchymal stromal cells (MSCs) is suggested as a main cause of altered bone repair with aging. We recently showed that in postmenopausal women undergoing cementless total hip arthroplasty (THA) aging, low bone mineral density (BMD) and age-related geometric changes of the proximal femur are risk factors for increased early migration and delayed osseointegration of the femoral stems. Extending these analyses, we have here explored how the *in vitro* osteogenic capacity of bone marrow MSCs from these patients reflects implant osseointegration, representing the patient's *in vivo* bone healing capacity. A total of 19 postmenopausal women with primary hip osteoarthritis (mean age 65 years, range 50–78) and well-defined bone quality underwent successful preoperative *in vitro* analysis of osteogenic capacity of iliac crest bone marrow MSCs as well as two-year radiostereometric (RSA) follow-up of femoral stem migration after cementless THA. In patients with MSCs of low osteogenic capacity, the magnitude of cumulative stem subsidence after the settling period of three months was greater ($p = 0.028$) and the time point for translational osseointegration was significantly delayed ($p = 0.030$) compared to patients with MSCs of high osteogenic capacity. This study suggests that patients with MSCs of low *in vitro* osteogenic capacity may display increased stem subsidence after the settling period of 3 months and thereby delayed osseointegration. Our study presents a novel approach for studying the biological progress of hip implant osseointegration and to verify the impact of decreased MSCs function, especially in patients with age-related dysfunction of MSCs and bone healing capacity.

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1. Introduction

Age-related dysfunction of mesenchymal stromal cells (MSCs) is emerging as the main cause of bone loss and altered repair capacity with aging (Kassem and Marie, 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. As in any bone healing process MSCs are essential in the progression of biological fixation, osseointegration, of cementless total hip arthroplasty (THA). If appropriate stem cells are not present, osteogenesis is inhibited (Kassem and Marie, 2011; Pountos et al., 2013; Kienapfel et al., 1999).

Total hip arthroplasty 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 North America, Australia and several European countries (Troelsen et al., 2013; Lehil and Bozic, 2014). Cementless THA relies on biological fixation of the implant to the surrounding bone, and is considered to take about 3–6 months. Despite the high number of cementless stems implanted annually, very little is known about biological factors actually affecting osseointegration of long-bone prostheses. Traditionally, implant and patient-related demographic factors (Fig. 1) have dominated the research focuses. Details on the biologic processes of osseointegration is mainly derived from dental implants, experimental animal and *in vitro* studies, and from autopsy retrievals (Davies, 1998; Goriainov et al., 2014; Sychterz et al., 2002; Engh et al., 1995). Osseointegration is defined as ingrowth and/or ongrowth of new bone to the surface of the implant (Branemark et al., 1977; Albrektsson et al., 1981), and includes a series of biological events regulated by a variety of cells and signaling molecules. Major part of the process depends on the actions of 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 under influence of growth factors and other biologically active molecules (*e.g.* cytokines).

In cementless THA, initial stability of the implant is critical for successful permanent biological osseointegration with the host bone

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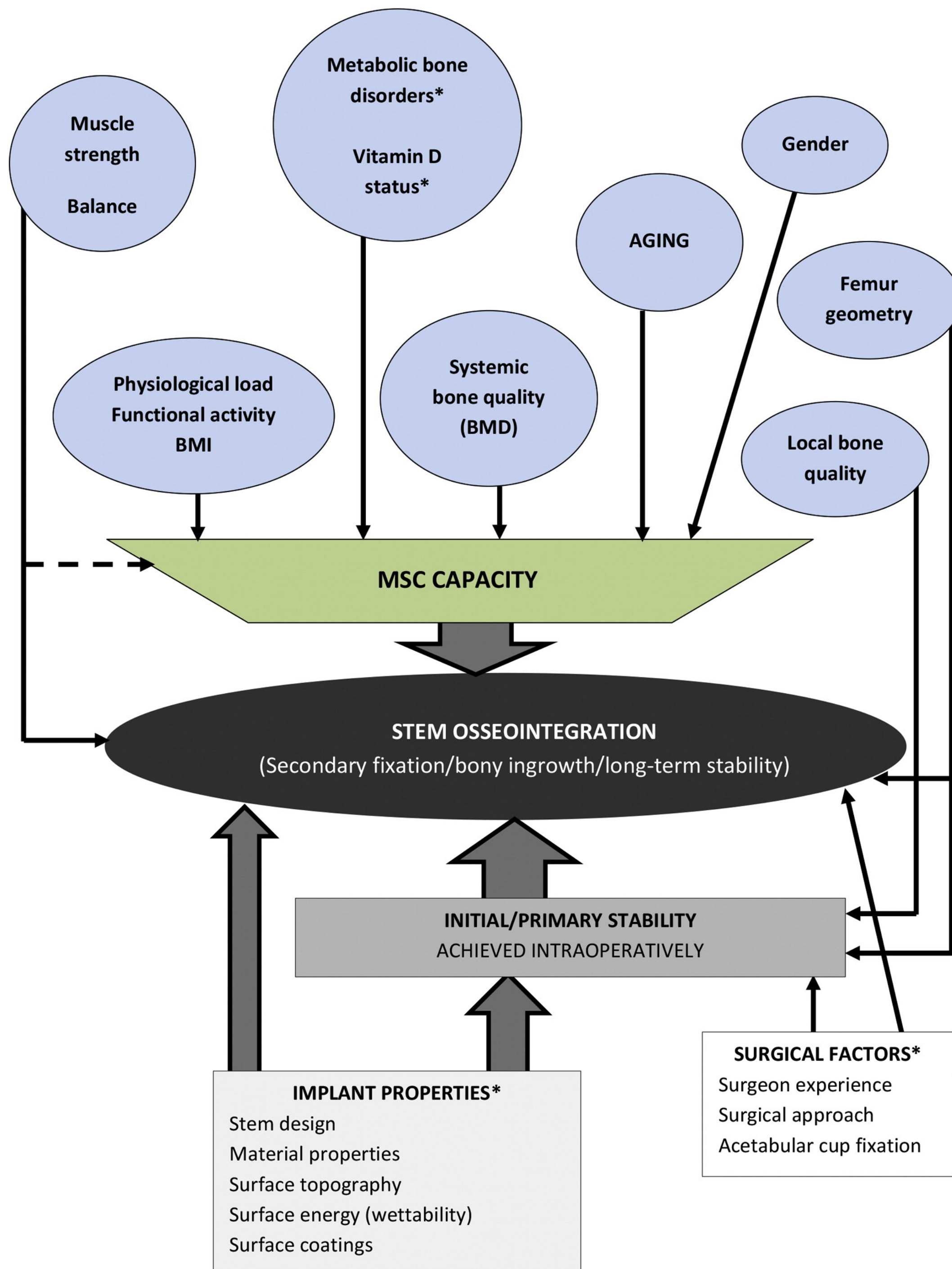

Fig. 1. Factors influencing osseointegration of cementless femoral stems. Requirements for proper biological fixation are; intimate initial contact between implant and host bone, minimal relative motion at the implant-bone interface, appropriate implant surface characteristics and osteogenic potential at the implantation site. Initial contact and minimal relative motion are prerequisite for the biological processes to take place. 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. Although implant parameters are crucial for a successful outcome, the cellular responses dictate the fixation process. Implant-related (light grey box) and patient-related (circles) factors have been extensively investigated while the role of MSCs is less understood. * indicate factors that can be controlled for. Based on (Goriainov et al., 2014; Mellon et al., 2013; Aro et al., 2012; Moritz et al., 2011; Alm et al., 2010). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Khanuja et al., 2011). This is affected by implant related factors (design, chemical composition, surface topography, coatings), surgical technique, and the quality of the surrounding bone bed (Goriainov et al., 2014; Khanuja et al., 2011; Engh et al., 1987; Mellon et al., 2013) (Fig. 1). 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). Traditionally stem stability/fixation is evaluated qualitatively from plain radiographs by identification of radiographic features including bone bridging, reactive lines, pedestal formation, calcar remodeling, radiolucent lines, implant position and migration exceeding 4 mm (Engh et al., 1990). Radiostereometric analysis (RSA) is a unique method allowing for monitoring of progression of implant osseointegration and anchorage in arthroplasty patients. The method

provides accurate and precise measurement of 3-dimensional implant micromotion relative to bone (Karrholm et al., 1997; Valstar et al., 2005). Hence, RSA measures stem fixation indirectly, as osseointegration is characterized by cessation of micromotion (Mann et al., 2012), and by applying RSA the time point for osseointegration can be identified. Although associated with high technical demands and expenses, RSA is a valuable research tool offering quantitative and early assessment of osseointegration, allowing for investigation of biological factors involved in the process in patient populations.

Although cementless THA was originally designed for middle-aged patients with normal or close to normal bone quality, current indications have expanded to include aged patients (Troelsen et al., 2013; Dutton and Rubash, 2008; Kelly et al., 2007; Makela et al., 2008). Postmenopausal women constitute a majority of cementless THA patients (Makela et al., 2008; Sadoghi et al., 2012). This patient group is prone to osteoporosis (Glowacki et al., 2003; Makinen et al., 2007) and may also exhibit qualitative and quantitative variations in their MSCs.

Despite the essential role of MSCs in osseointegration, there is still little knowledge on whether intrinsic properties of MSCs, such as osteogenic differentiation and the ability to lay down mineralized matrix correlate with the outcome of an arthroplasty. The relationship between *in vitro* properties of MSCs and biological implant fixation has, to our knowledge, never been explored in humans. Using RSA, we have investigated the impact of different bone quality parameters on the healing of cementless THA in postmenopausal women with primary hip osteoarthritis (OA). We recently showed that aging, low bone mineral density (BMD) and age-related geometric changes of the proximal femur are risk factors for early migration and delayed osseointegration of cementless femoral stems (Aro et al., 2012), while the quality of intertrochanteric cancellous bone was less important for the initial stem stability (Moritz et al., 2011). Extending our previous analysis, we have here investigated how *in vitro* assayed osteogenic capacities of patient's MSCs reflect in implant healing. We hypothesized that individual variation in MSC capacity may influence the implant healing process, and patients with diminished osteogenic capacity of their MSCs would show increased implant migration and delayed osseointegration.

2. Patients and methods

2.1. Patient enrolment and surgery

The study protocol was approved by the Ethics Committee of the Hospital District of Southwest Finland and the study was conducted in accordance with the Code of Ethics of World Medical Association (Declaration of Helsinki). All patients provided written informed consent prior to enrollment. The original study population consisted of 61 consecutive generally healthy female patients <80 years of age with advanced primary hip OA scheduled for cementless THA surgery (Makinen et al., 2007). As previously reported in detail (Makinen et al., 2007), patients were selected for enrollment through careful screening including blood chemistry, and dual-energy x-ray absorptiometry (DXA) measurements of BMD (at the lumbar spine, both proximal femurs, and the distal non-dominant forearm) in order to exclude patients with undiagnosed metabolic bone disorders, including severe osteoporosis (T-score < -3.5 at any anatomical site). The preoperative DXA measurements were used as indicator of baseline systemic skeletal status; patients were classified as having normal BMD (T-score > -1.0), osteopenia (T-score between -1.0 and -2.5) or osteoporosis (T-score < -2.5) (Makinen et al., 2007). None of the patients included in the current analyses were on osteoporosis treatment before or after THA (Fig. 2). All patients received vitamin D and calcium supplementation during the two-year follow-up. For subjective evaluation of functional recovery, validated disease-specific scoring systems for assessment of pain and physical function were applied at baseline and each follow-up. Western Ontario and McMaster arthritis index (WOMAC) was applied as a disease-specific scoring for patient-related outcome, and Harris hip score (HHS) was applied as a physician assessment of clinical outcome.

The patients underwent cementless THA using an RSA-marked hip implant with proximal hydroxyapatite-coating and ceramic-ceramic bearing surfaces (Anatomic Benoist Girard II, ABG II, Stryker), with subsequent two-year RSA follow-up of implant healing as described previously (Aro et al., 2012). For analyses of MSCs, the patients underwent bone marrow aspiration from the posterior iliac crest under spinal anesthesia prior to surgery. Of the 53 eligible patients (Makinen et al., 2007), MSCs were isolated, cultured and analyzed for osteogenic differentiation capacity from bone marrow samples of 30 patients (Supplementary Table 1).

2.2. Radiostereometric analysis of femoral stem migration and osseointegration

Three-dimensional migration of the anatomically shaped RSA-marked femoral stem (Fig. 3A) was evaluated using uniplanar RSA imaging technique at baseline (within 7 days after surgery) and at 3, 6, 12 and 24 months after surgery (Fig. 3B-C). RSA examination was done using UmRSA 6.0.3.7 software (RSA BioMedical Innovations AB, Umeå, Sweden). Migration of the stem was analyzed as translation (linear movements) and rotation (angular movements) along three axes (y, x, z) (Fig. 3D). Translational migration along the y-axis (*i.e.* stem subsidence) and rotation around the y-axis were used as main parameters. As an expression of three-dimensional translational and rotational movements, the translation vector and the rotation vector were calculated, respectively (Kaptein et al., 2007). Analyses were performed according to the RSA guidelines (Karrholm et al., 1997; Valstar et al., 2005).

The traditional RSA measurements describe change in implant position at each follow-up time point compared to baseline. In addition, we calculated the cumulative migration detected between follow-up time points (3 to 6 months + 6 to 12 months + 12 to 24 months) as a measure of total distance of migration. Since most cementless stem designs display some degree of migration, mostly subsidence, during the settling period of the first 3 postoperative months, we chose the 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.

RSA data was further used to assess stem osseointegration (*i.e.* implant stability as sign of complete healing) at the individual patient level (Aro et al., 2012). 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 measurements (0.42 mm for y-translation, 0.40 mm for translation vector, 1.81 degrees for y-rotation, and 1.32 degrees for the rotation vector), defined by double examinations (Moritz et al., 2011).

The two-year postoperative RSA follow-up of femoral stem migration was successfully completed from 19 patients (age 65 ± 8, 50–78 years), including four with normal systemic BMD, 12 with osteopenia and three with osteoporosis. All patients had femur morphology corresponding to Dorr type A ($N = 12$) or type B ($N = 7$). Eleven patients were excluded from the RSA follow-up analysis due to; initiation of antiresorptive drug therapy for severe osteoporosis ($N = 9$), surgical complication ($N = 1$), or absence of RSA markers ($N = 1$) (Fig. 2).

2.3. Isolation and culture of bone marrow MSCs

MSCs were isolated from the BM aspirates (3–5 mL) and cultured as described previously (Alm et al., 2010; Alm et al., 2012). Briefly, density gradient isolated mononuclear cells were seeded at 80,000 cells/cm^2 and cultured in phenol red free alpha-MEM (Gibco Invitrogen) supplemented with 10% fetal calf serum (Gibco Invitrogen cat. #16000) and penicillin-streptomycin (Gibco Invitrogen) as the basal medium. Non-

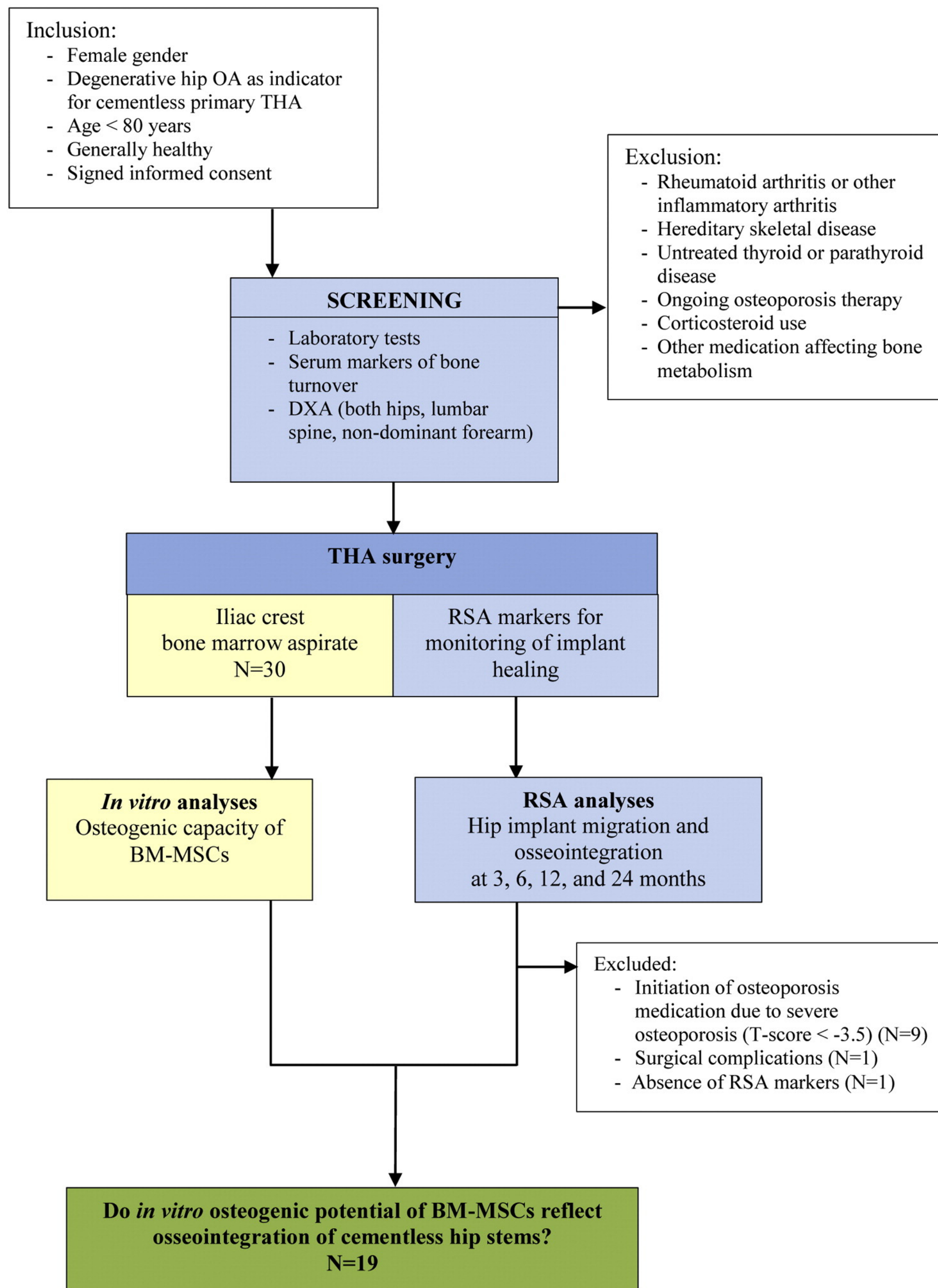

Fig. 2. Study design.

adherent cells were discarded after 48 h, and sub-confluent cultures were re-plated at 1000 cells/cm^2 and expanded through several passages. Isolated MSCs were characterized (Alm et al., 2010) by successful expansion and differentiation into osteoblasts and adipocytes. The MSC phenotype (CD105$^+$, CD73$^+$, CD90$^+$, CD45$^-$ and CD14$^-$) and chondrogenic differentiation capacity was confirmed in a subgroup by immunocytochemistry and micromass cultures, respectively, according to the ISCT minimal criteria (Dominici et al., 2006). Proliferative capacity was evaluated by calculating the number of cell population doublings (PDs) at each passage using the formula log N/log 2 (N is the number of cells at harvest divided by the number of cells plated).

2.4. Osteogenic differentiation

For assessment of osteogenic differentiation capacity, passage 1–3 MSCs were seeded at 2500 cells/cm^2 in four replicate wells and cultured in basal medium supplemented with 10 mM sodium β-glycerophosphate (Merck) and 0.05 mM ascorbic acid-2-phosphate (Sigma-Aldrich). During the first 7 days of induction, 100 nM dexamethasone (Sigma-Aldrich) was used as an additional supplement (Alm et al., 2012). Parallel cultures in basal medium served as control. Osteogenic differentiation was demonstrated by analyzing for alkaline phosphatase (ALP). ALP activity was measured spectrophotometrically after 2 weeks in osteogenic culture medium. ALP activity was converted to units/μg protein. After 4–5 weeks in osteogenic medium, cultures were stained for ALP (ALP kit, Sigma Diagnostics, St. Louis, MO, USA). As a measure of complete differentiation into functional osteoblasts, extracellular matrix mineralization was analyzed with von Kossa staining and by spectrophotometric measurement of deposited calcium using a commercial kit (Roche Diagnostics). ALP and von Kossa stainings were quantified histomorphometrically using an automated image analysis as described previously (Alm et al., 2012). Briefly, the stained plates

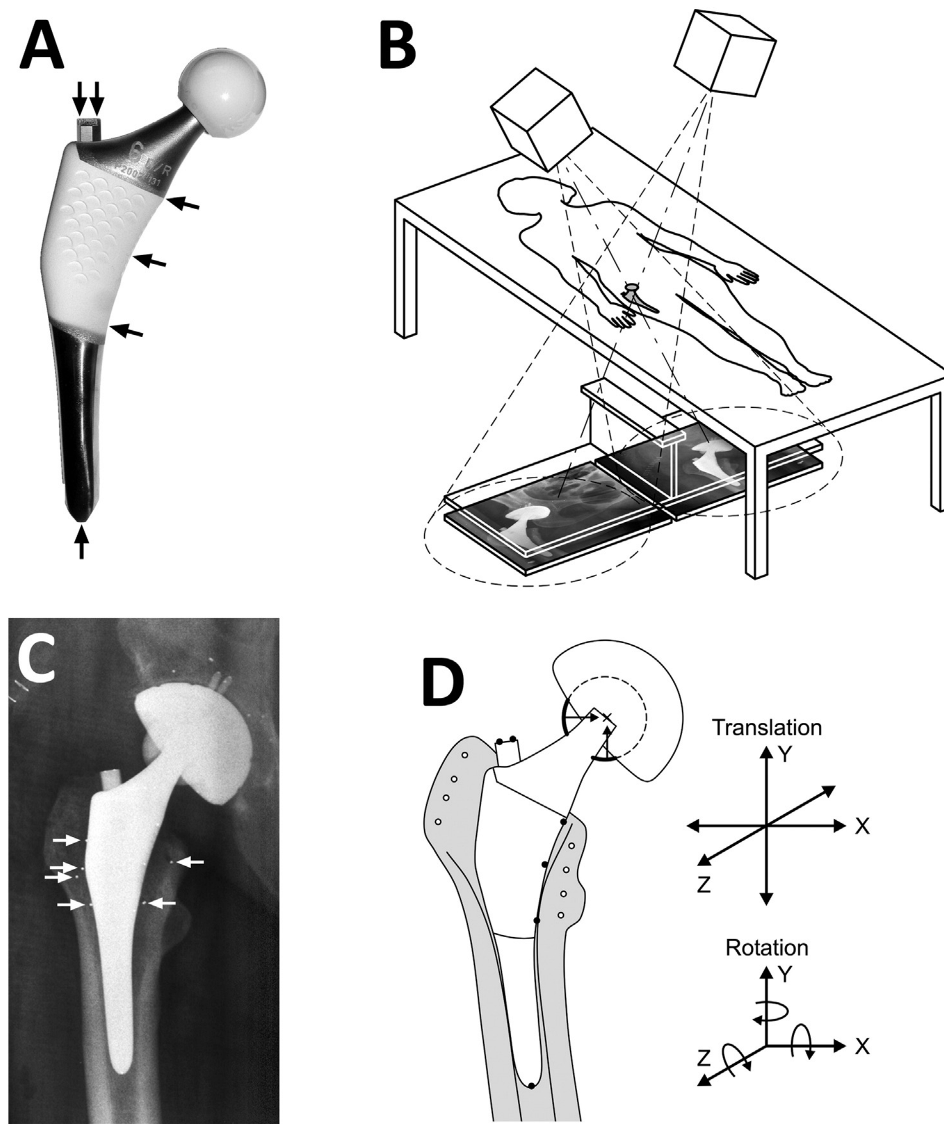

Fig. 3. Radiostereometric analysis of the cementless femoral stem. (A) The ABG-II titanium alloy stem with proximal hydroxyapatite coating, polished distal part and ceramic head. The six tantalum RSA markers are indicated with arrows. (B) Schematic drawing of radiographic examination of the operated hip with simultaneous exposure of two X-ray units. UmRSA calibration cage with X-ray plates under the examination table. (C) Postoperative radiograph of a prosthesis with six 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, along with the coordinate system for RSA analysis of three-dimensional stem migration in relation to the femur (D).

were scanned using a flatbed scanner with a transparency adaptor (HP ScanJet 5370C) and a custom-designed holder for accurate and reproducible positioning of the plates during scanning at 600 dpi. Transparency exposure adjustments were kept constant to generate images of equal intensity, and images were saved as 24-bit color images in TIF format. Images were analyzed using the automated image analysis software and the stained area (cm^2) was converted to percentage of the total area and presented as mean percentage stained area from four replicate wells.

2.5. Assessment of osteogenic capacity of MSCs

MSCs from all donors ($N = 30$, Supplementary Table 1) demonstrated osteogenic differentiation, although at varying degree, but the level of mineralization was generally low and complete mineralization was detectable only for MSCs from part of the patients (Supplementary Table 2, Supplementary Fig. 1). From 13 patients out of 30, 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. Therefore, for the purposes of analyzing the relationship between *in vitro* osteogenic differentiation capacity of MSCs and hip implant migration ($N = 19$, Fig. 2), patients were classified as having MSCs with high or low osteogenic (OB) capacity based on the combined ALP and mineralization outcome. First, the patients were ranked for the 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. Second, the patients were ranked for the mineralization capacity of their MSCs, *i.e.*, von Kossa stained area, using the same method. Thereafter, the OB-capacity groups were obtained by calculating the sum of the ALP and the mineralization indexes for each patient. The low OB-capacity group was defined as patients with a combined index between 2 and 4 ($N = 12$), and the high OB-capacity group was defined as patients with a combined index of 5 and 6 ($N = 7$). This strategy ensured the high OB-capacity group did not include any patient with low ranking

Table 1
Demographic and clinical characteristics of postmenopausal female THA patients ($N = 19$).

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 for evaluation of hip OA (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.

in either of the MSC quantification methods. See supplementary material for details on the classification process.

2.6. Statistical analysis

The data is shown as mean ± SD (range). A significance level of 0.05 was applied. Comparisons between the low and high OB-capacity groups were done using Student's *T*-test after confirming that data fulfilled the criteria (normal distribution and equal variances). Possible influence of confounding demographic and clinical factors on the outcome was ruled out by separate linear correlation analyses with the study main parameters (MSC data and RSA data, respectively). In analysis of mean change in femoral stem position over time in the two subgroups, it was ensured that the RSA data did not include outlier values. Prior to all 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.

3. Results

3.1. RSA measured change in femoral stem position

The two-year postoperative RSA follow-up of femoral stem migration was successfully completed from 19 patients (Tables 1 and 2) who were classified as having MSCs with high ($N = 7$) or low ($N = 12$) osteogenic (OB) capacity based on the combined ALP and mineralization outcome. 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 3, Fig. 4A). A similar trend was seen for the three-dimensional translation (translation vector). However, in the low OB-capacity group, stem subsidence significantly exceeded the detection limit (0.42 mm) for stem migration by 3 months ($p = 0.020$), 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 rotation (rotation vector), were of similar magnitude in the two OB-capacity groups (Table 3, Fig. 4B).

3.2. Cumulative femoral stem migration after the 3 months settling period

In the whole group of 19 patients, the calculated cumulative migration from 3 to 24 months ranged between 0.1 and 2.2 mm (mean 0.6 ± 0.5) along the y-axis (stem subsidence) and between 1.1 and 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 ($p = 0.028$)(Fig. 5A), as was the magnitude of cumulative three-dimensional translation (translation vector, $p = 0.043$)(Fig. 5B). The cumulative rotation along

Table 2
Iliac crest BM-MSC characteristics of postmenopausal THA patients ($N = 19$).

	Mean ± SD	Range
BM aspirate cellularity		
MNCs (10^6)/mL bone marrow	3.6 ± 6.4	0.2–30
CFU/BM MNCs (%)	0.003 ± 0.002	0.001–0.008
Growth capacity (maximum value passage 1–3)		
Max population doublings	3.9 ± 1.4	1.0–6.9
Max population doubling rate (PDs/day)	0.12 ± 0.07	0.02–0.27
Osteogenic differentiation capacity		
ALP activity (U/g protein)	12 ± 5	7–23
ALP stained area (%)	34 ± 27	1–90
von Kossa stained area (%)	12 ± 15[a]	0–52
	32 ± 13[b]	5–52
Ca (mmol/L)	0.16 ± 0.14	0.07–0.62

[a] All donors, including zero values.
[b] Only values >0 included.

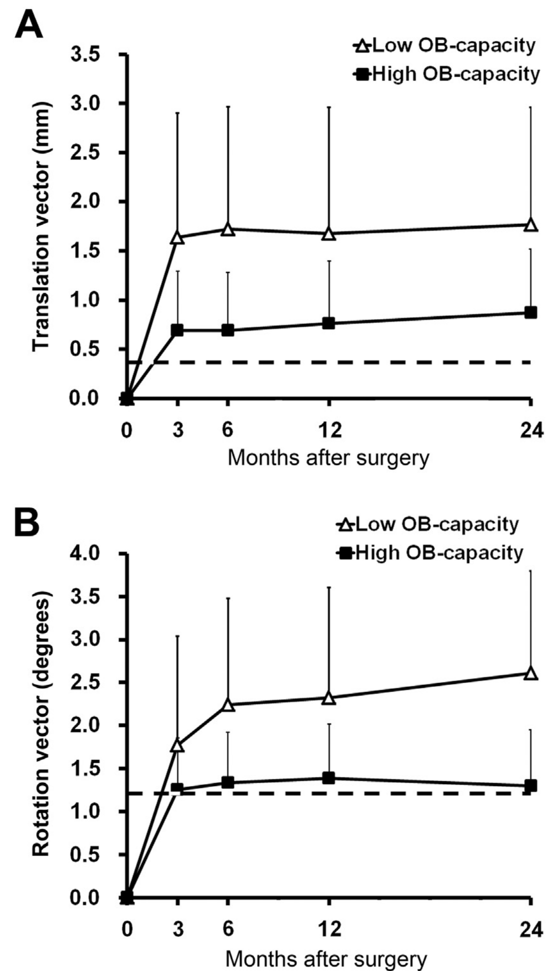

Fig. 4. RSA-measured change in femoral stem position up to 24-months after surgery. Three-dimensional (A) translation (translation vector) and (B) rotation (rotation vector) of the cementless femoral stem in female patients with MSCs of low or high osteogenic capacity. In patients with MSCs of low OB-capacity, translational change in stem position significantly ($p = 0.015$) exceeded the detection limit (dotted line), indicating migration. Dotted lines indicate the parameter-specific detection limits. Data presented as mean with SD.

the y-axis and three-dimensional rotation (vector) from 3 to 24 months varied widely between subjects, with corresponding magnitude in the two OB-capacity groups (Fig. 5C-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 1). In addition, the linear correlation analyses between the dependent variables and possible confounders showed no interactions.

3.3. In vitro osteogenic capacity of MSCs as predictor of time point for 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-

Table 3
RSA-measured femoral stem migration (change in stem position) in patients with high and low OB-capacity of their bone marrow MSCs.

	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.

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 between the groups. The estimated probability for osseointegration within 3 months was 57% in the high OB-capacity group and 25% in the low OB-capacity group (Supplementary Table 3).

The RSA-measured stem migration had no impact on the 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 1). Both groups showed a significant improvement of the 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 experiments (passage 2 and 3).

3.4. *In vitro osteogenic properties of MSCs correlate with clinical bone quality parameters*

To further investigate the relevance of *in vitro* testing of osteogenic capacity of bone marrow MSCs in relation to bone health of the donors, analyses were performed including all the study patients who had successful iliac crest bone marrow aspirate before THA surgery ($N = 30$) (Fig. 2, Supplementary Table 1). In linear correlation analyses, ALP stained areas was used as a measure of *in vitro* osteogenic differentiation capacity (Fig. 6). MSCs from subjects with low preoperative T-score showed decreased osteogenic differentiation ($r = 0.42$, $p = 0.019$, $N = 30$)(Fig. 6A). There was an inverse relationship between *in vitro* ALP expression and serum levels of vitamin D (25(OH)D)($r = -0.42$, $p = 0.021$, $N = 30$) (Fig. 6B). Further, ALP levels in MSC cultures correlated with serum levels of bone formation marker ALP ($r = 0.38$, $p = 0.045$, $N = 28$) (Fig. 6C) and bone resorption marker TRACP-5b ($r = 0.50$, $p = 0.006$, $N = 28$) (Fig. 6D).

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*

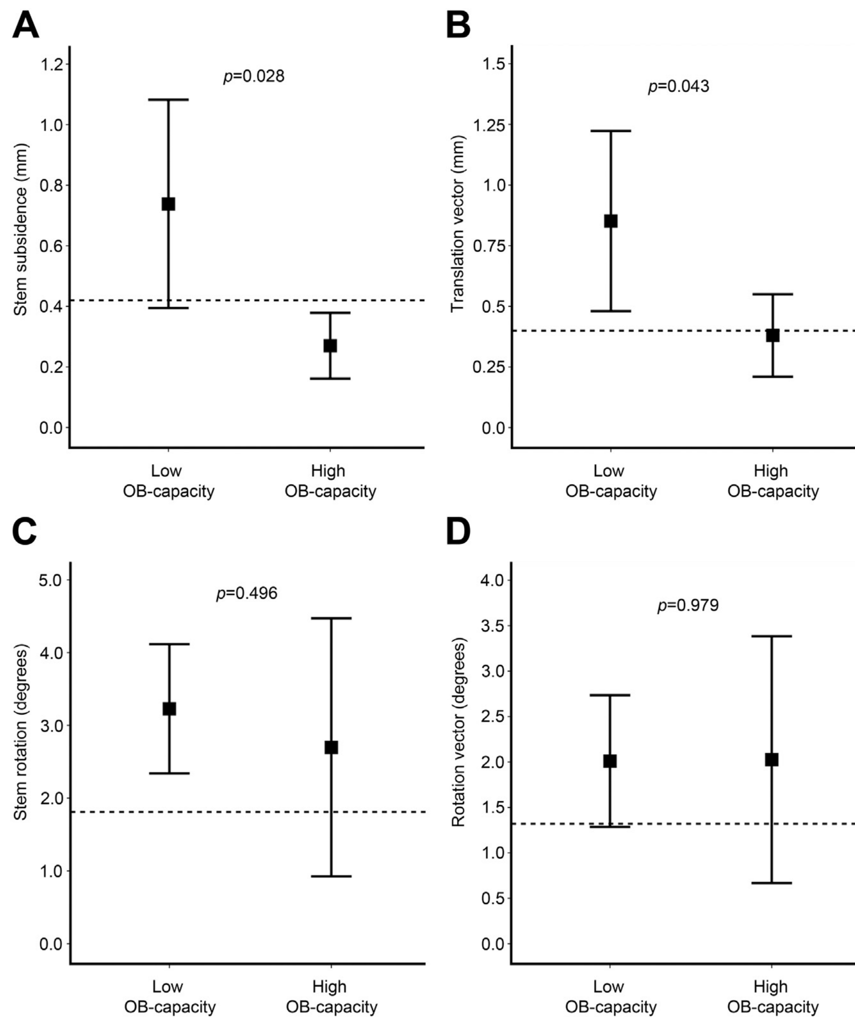

Fig. 5. Cumulative stem migration from 3 to 24 months. In patients with MSCs of low osteogenic capacity ($N = 12$) the magnitude of cumulative migration (A) along the y-axis (stem subsidence) and (B) in three-dimensional translation (translation vector) was significantly higher compared to the high OB-capacity group ($N = 7$). The magnitude of cumulative (C) rotational migration around the y-axis (stem rotation), and (D) three-dimensional rotation (rotation vector) was similar in the two OB-capacity groups. Data presented as mean with 95% confidence interval. p = two-tailed p-value from Student's T-test. Dotted lines indicate parameter-specific detection limits, *i.e.*, limits of detectable migration.

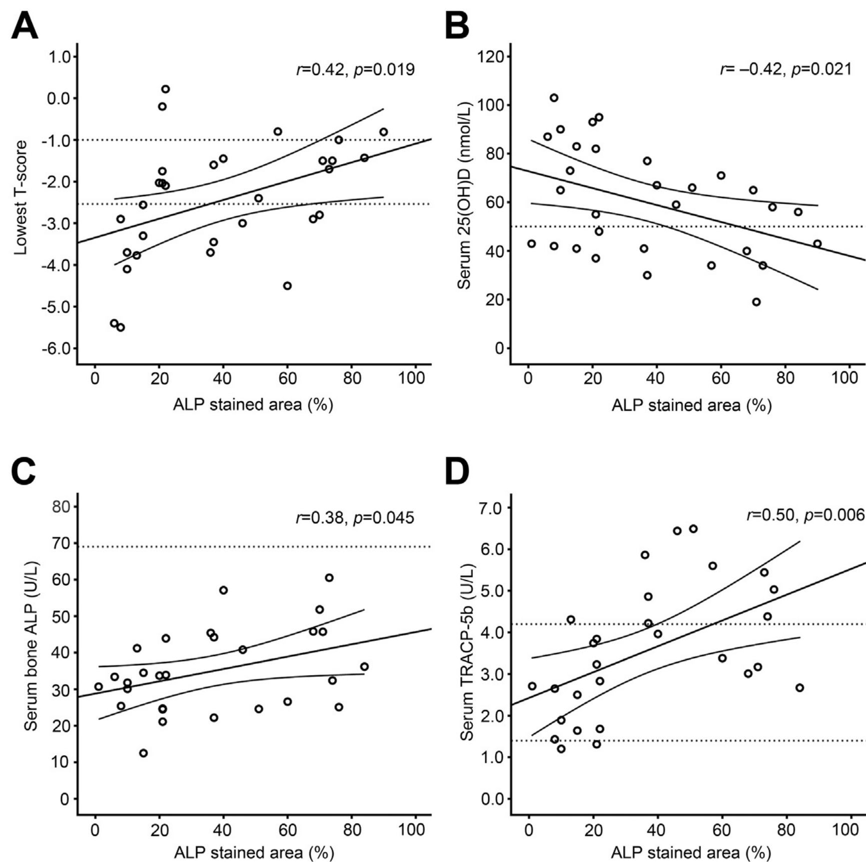

Fig. 6. Correlations between clinical parameters of bone quality and *in vitro* osteogenic differentiation capacity of the patients' bone marrow MSCs. The osteogenic differentiation capacity of the MSCs, represented by expression of alkaline phosphatase (ALP), correlated significantly with (A) the DXA-measured lowest *T*-score (lumbar spine, proximal femurs, non-dominant forearm) of the patients ($N = 30$). Dotted lines indicate *T*-score limit for osteopenia (upper line) and osteoporosis (lower line), respectively. (B) There was an inverse correlation between MSC-expressed ALP and serum levels of 25(OH)D ($N = 30$). Dotted line indicate limit for vitamin D insufficiency. Osteogenic differentiation capacity of MSCs also correlated with serum levels of (C) bone formation marker bone ALP ($N = 28$), and (D) serum levels of resorption marker TRACP-5b ($N = 28$) (D) (dotted lines indicate normal ranges). r = Pearson correlation coefficient; p = two-tailed p-value.

mineralization ($N = 17$)(lowest T-score $- 1.9 \pm 1.1$, $p = 0.044$). 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 or BMI in the present patient population.

4. Discussion

This study explored how *in vitro* osteogenic capacity of bone marrow MSCs from postmenopausal women undergoing cementless THA reflects implant osseointegration, representing the patient's *in vivo* bone healing capacity. RSA monitoring of three-dimensional migration of the cementless femoral stems confirmed our hypothesis, demonstrating that patients with MSCs of low *in vitro* osteogenic capacity displayed increased stem subsidence after the settling period of 3 months, and thereby delayed osseointegration.

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. A remaining question is whether osseointegration in patients undergoing cementless THA is mirrored by the *in vitro* ability of their MSCs to differentiate into bone cells and mineralize under osteogenic culture conditions. Development of the highly accurate RSA imaging technology has revolutionized our capabilities for monitoring the process of implant healing (Valstar et al., 2012; de Vries et al., 2014). By allowing measurements of micromotions not detectable with other imaging methods, stability/fixation of the stem can be indirectly measured, and RSA makes it possible to investigate the impact of unexplored biological elements, including MSCs, on bone healing in cementless THA patients, reaching beyond the conventional implant related and biomechanical factors. In order to demonstrate 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 (<65-years old) male and female patient populations with good skeletal health RSA-migration is minimal or not detectable. Postmenopausal aging women represent this type of an ideal study population not only for the scientific purpose but for the clinical relevance based on the fact that: (1) postmenopausal women represent the largest group of patients undergoing THA (Makela et al., 2008; Sadoghi et al., 2012), (2) they are prone to impaired skeletal quality with increased risk of complications including delayed osseointegration (Vandamme et al., 2011; Marco et al., 2005) and periprosthetic fractures (Sidler-Maier and Waddell, 2015), and (3) they display an age-related decrease of MSC capacity (Kassem and Marie, 2011; Baker et al., 2015). In the current study, the selected patients were postmenopausal women who suffered from primary hip OA but were otherwise healthy and without bone-affecting medication. They underwent standardized surgical procedure performed by a single experienced orthopedic surgeon, receiving the ABGII anatomically-shaped femoral stem size-fitted to obtain optimal press-fitting, further limiting possible confounding factors.

We believe that our results on increased femoral stem subsidence in patients with decreased *in vitro* OB-capacity of their MSCs 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, including age, systemic and local BMD, rate of bone turnover, S-25(OH)D status, and 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 the whole group of recruited patients ($N = 30$) showed correlations between OB-capacity and clinical bone quality parameters, such correlations were not present for the 19 patients constituting the main study population. As we previously demonstrated (Aro et al., 2012), the geometry of the proximal femur is a significant factor affecting stem migration. Importantly, proximal femur geometry was ruled out as a confounding factor in the current analysis, and the study constituted only patients with Dorr type A and B shaped femurs equally distributed between the two OB-capacity groups.

It is important to emphasize that 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) 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. The functional recovery of the patients was also uneventful and there was no need for revisions. These findings are in well agreement with recent registry studies showing excellent 10-year survival (99% for the ABG stem) for cementless femoral stems of current designs (Hailer et al., 2015). Thus, the decreased OB-capacity of MSCs seen in our patient group is associated with subclinical stem migration and delayed stabilization, resembling the clinical event of osteoporotic fracture healing. In experimental models of osteoporosis (Egermann et al., 2008) the rate of bone fracture healing is decreased, while in patients, fractures heal in a fairly predictable manner despite osteoporotic conditions. However, this does not diminish the scientific value of the current results. On the contrary, our study 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 biological process of implant healing, particularly in patients with age-related dysfunction of MSCs and decreased bone healing capacity. *In vitro* and experimental animal studies have demonstrated the significant influences of implant topography and surface properties on MSCs attachment and differentiation and thereby implant fixation (Goriainov et al., 2014). Microrough surfaces stimulates osteogenic differentiation of MSCs through activation of the non-canonical Wnt signaling pathway (Olivares-Navarrete et al., 2011), while hydroxyapatite coating is a strong activator of the canonical Wnt signaling pathway (Thorfve et al., 2014). Our clinical study further supports efforts focusing on improving implant fixation through surface modifications affecting MSCs (Goriainov et al., 2014), which could compensate for decreased OB-capacity of the MSCs.

It can be argued that the use of cemented fixation could provide an attractive alternative in patients at risk of decreased MSC capacity. 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. Clinical reports indicate that osteoporosis is a risk factor for late mechanical loosening of cemented hip prostheses (Broden et al., 2015). In our opinion, 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.

Expression of ALP is a measure of early osteoblastic differentiation, while mineralization is a late marker of complete differentiation into matrix producing osteoblasts. Since both properties are essential in terms of osseointegration, we divided the patients into low and high OB-capacity groups based on the combined ALP and mineralization outcomes for the purposes of analyzing the relationship between *in vitro* osteogenic capacity of MSCs and hip implant migration. The ranking strategy for indexing the 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* properties of the patients' MSCs. 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.

A number of signaling molecules are involved in MSC survival, proliferation, recruitment, activation and differentiation. Individual variation in endogenous levels of such factors could contribute to differences in both *in vitro* properties of MSC as well as *in vivo* properties of MSCs affecting the healing responses upon THA surgery, although clinical evidences are limited. Increased serum levels of BMP-2 have been found in patients with knee OA compared to healthy controls (Liu et al., 2015). Such variations in BMP-2 levels cannot be ruled out as an underlying contributing factor to the observed differences in OB-capacity and osseointegration in the current study. However, patients with normal and delayed fracture healing have similar levels of circulating BMPs (van Baardewijk et al., 2013). Circulating level of TGF-β1 is another crucial factor affecting MSC differentiation, demonstrated to increase after fracture (Sarahrudi et al., 2011) and displaying different time-dependent levels in normal compared to delayed fracture healing (Zimmermann et al., 2005). Currently, there are no corresponding data available regarding THA.

The aim of this study was not to investigate the correlation between MSC properties and demographic parameters; rather this was performed as secondary, *post hoc*, analyses including MSCs from the whole study population of 30 THA females. The 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 clinical 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. (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. The weak correlations in the current study (explaining 14–25% of the MSC ALP expression) and in the study by Zhou et al. (Zhou et al., 2012) (explaining 13–24%) probably reflect the discrepancy between artificial and simplified *in vitro* conditions and the true complex *in vivo* situation. As a general observation, cell yield, expansion capacity and osteogenic differentiation properties of MSCs from the current group of postmenopausal THA females varied widely and were approximately 50% compared to MSCs from younger premenopausal females (mean age 40 $\pm$ 16, range 19–60) from our previous studies (Alm et al., 2010).

This study carries both strengths and limitations. The homogenous and carefully screened female population brought strength to our study, along with application of the sophisticated RSA method for monitoring of implant micromigration, in combination with analysis of individual patients' MSCs. However, the RSA method carries certain limitations, mainly related to the technical demands and high costs. Every aspect of the method has to be meticulously followed in order to obtain valid outcomes, and it is time-consuming. Our group has paid great attention to standardize the applied RSA technology using a phantom model (Makinen et al., 2004; Madanat et al., 2014), and the CE-certified implant was custom-modified by a leading international manufacturer in order to assure accuracy and precision. RSA imaging and analysis were performed by two trained technicians and an

experienced bioengineer. Presenting and interpreting RSA data has its own pitfalls. Analyzing mean values, according to the RSA guidelines (Valstar et al., 2005), is useful when evaluating performance of new implant designs or investigating the impact of medical intervention, but provide limited information at the individual patient level. 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. We therefore extended the traditional RSA data analyses by assessing stem migration over time separately for each individual patient for identification of time point of osseointegration/stabilization, and by evaluation of cumulative distance of migration taking place after the initial 3 months settling period. For iliac crest bone marrow aspirations, standardized techniques were adopted (Muschler et al., 1997) and we have optimized the MSC culturing protocols (Alm et al., 2012). Through the approach to divide patients into low and high OB-capacity groups the limited statistical power due to large variability in the MSC data was evaded. Large inter-individual variability is a well-documented reality associated with human MSCs. 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, especially in this type of prospective study. This study did not include gene expression analysis of the MSCs. Such analysis could possibly have exposed differences in expression patterns of key osteogenic genes (RUNX2, OSX, ALP, COL1, OCN) or even frequencies of committed MSCs from different patients, contributing to understanding the underlying mechanisms to the observed low and high *in vitro* osteogenic capacities. However, gene expression levels do not always correspond to the actual protein levels, and do not reflect the osteogenic functionality of the cells. This study do not delineate the possible impact of gender, type of cementless stem or cement fixation on the relation between MSC capacity and stem osseointegration. The impact of the specific stem design (ABGII) used in this study should not be neglected. With straight non-anatomical designs relying on 3-pont fixation, or tapered stems the migration patterns would most likely be different, and the correlation to MSC capacity could also show different trends. Since the physical activity level of the patients was not analyzed in the current study, possible underlying effects of this factor on both the RSA data and MSCs capacity levels cannot be evaluated. 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 ($N = 30$) and the number of patients completing the 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 subgroups were undersized. Further research is warranted for more detailed analyses of underlying and contributing mechanisms.

5. Conclusions

In this prospective clinical study we analyzed the preoperative *in vitro* osteogenic capacity of bone marrow MSCs from postmenopausal women undergoing cementless total hip replacement, in combination with a 2-year postoperative radiostereometric monitoring of their femoral stem migration. We assumed that the rate of hip stem osseointegration partly reflects the patients' MSC capacity, along with other known factors affecting osseointegration (Fig. 1). The results demonstrated increased cumulative migration and delayed osseointegration of femoral stems in patients with low MSCs osteogenic capacity. Our study presents a novel approach for studying the biological progress of hip implant osseointegration and to verify the impact of decreased MSC function. Since the RSA method allows detection of subclinical micromotions of cementless hip implants, subjects with impaired implant healing can be identified. Individualized aspects are emerging in orthopaedic medicine, where MSCs present a key tool in tailoring future improved implant osseointegration (Lewallen et al., 2014). Results from our previous studies suggest that (female) patients scheduled for cementless THA should be more carefully screened for systemic BMD status (DXA), secondary causes of osteoporosis (laboratory tests), and geometric femoral changes of the affected hip in order to identify patients at risk of delayed implant healing (Makinen et al., 2007; Aro et al., 2012). Extending these clinical assays, the current study suggests that analysis of autologous MSC properties is an aspect to consider, especially in patients with decreased bone quality, and/or in patients scheduled for more challenging bone reconstructive surgeries, where “boosting” of bone formation can be of relevance. Considering current efforts focusing on boosting, mobilization and homing of endogenous MSCs for enhancement of bone repair (Herrmann et al., 2015), preoperative *in vitro* evaluations of the patients' MSCs for guidance of appropriate approach for the individual patient could be useful. Both RSA imaging technique and MSC analysis are internationally established and ethically approved. However, these methods are technically challenging, time-consuming, expensive and labor requiring, especially in prospective clinical research settings of surgical implant patients. The current study encourages further research exploring the role of MSC in bone and implant healing with focused targets.

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Conflicts of interest

The authors report no conflict of interest.

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