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ANALYSIS OF CIRCULATING MESENCHYMAL PROGENITOR CELLS AND SELECTED CYTOKINES DURING BONE REGENERATION

ANALYSIS OF CIRCULATING MESENCHYMAL PROGENITOR CELLS AND SELECTED CYTOKINES DURING BONE REGENERATION

PhD-thesis 2010

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PREFACE

This thesis is the result of studies performed at the Department of Orthopaedic Surgery, the Research Laboratory Department of Haematology, Herlev Hospital Copenhagen University and Aalborg Hospital Innovation and Science Center, Aarhus University. Supervisors were Professor Hans E Johnsen, The Department of Haematology, Medical Center, Aalborg Hospital, Aarhus University; Associate Professor Jens Kastrup Medical Department B, The Heart Centre, Rigshospitalet, University of Copenhagen; Associate Professors Bo Sanderhoff Olsen and Gunnar Schwarz Lausten, The Department of Orthopaedic Surgery, Herlev Hospital University of Copenhagen.

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SUMMARY

Background

The existence of primitive mesenchymal progenitor cells in the non-haematopoietic compartment of the postnatal bone marrow has been documented in experimental animal and human studies. These progenitor cells have a preserved ability to differentiate into several kinds of mesodermal and neuroectodermal tissue as bone, cartilage, fat, skin, neuronal- and fibrous tissue, muscle and blood vessels. In spite of their meso-, neuro- and endodermal differentiation potential, this cellular compartment is termed mesenchymal progenitor cells (MPC). Investigations on differentiation of such progenitor cells in vitro and animal experiments have raised the theoretical possibility of new cell based treatments for degenerative diseases.

Less focus has been on the behaviour and characterization of circulating progenitor cells during normal tissue homeostasis and regeneration of traumatized tissue. Recent technological improvements have established proof of the existence of a population of blood born progenitor cells with the ability to differentiate into several types of mesodermal derived tissues. This progress has made this project relevant attempting to identify, characterize and enumerate blood born progenitor cells and identify molecules of potential significance for tissue regeneration following traumatic bone lesions.

Objectives and delimitations

The objectives of this project were to establish a clinical human model for studies to understand posttraumatic changes in circulating MPCs and biological active molecules involved in bone regeneration. The project has focused on traumatic bone fractures or planned surgical replacement of the hip joints, as a novel clinical model to study consecutive blood samples for associated variables including highly selected molecules and predefined low frequent non-haematopoietic mononuclear cells (MNC). This model seems qualified to investigate the cellular and humeral involvement in posttraumatic regeneration, because hip fractures heal through regeneration of new blood vessels, cartilage and bone, while artificial hip joints are fixated with toxic bone cement and do not produce any appreciable bone formation.

Hypotheses and aims

It is the hypotheses of this PhD project that circulating tissue specific bone and endothelial progenitor cells and associated molecules can be detected in the blood during initial bone regeneration following two clinical bone lesion models of traumatic ankle- or hip fractures or planned hip surgery. The analytic strategy included study of circulating cells and plasma with the following aims:

To identify and enumerate predefined subpopulations of bone and endothelial progenitors by multi parametric flow cytometry analysis of the MNC fraction in peripheral blood.

To screen the general gene expression in circulating MNCs from model patients by microarray analysis to identify differences in gene expression, determine functional gene clusters involved in bone regeneration and pinpoint the presence of specific benchmark genes, with known relation to bone regeneration.

To describe blood levels of selected circulating cytokines and growth factors by ELISA known to be correlated to bone regeneration.

It was the expectation that the analysis of changes of MPC levels could describe the temporal cellular contribution of specific subsets of progenitor cells to bone regeneration. The phenotype and varying number of such specific cellular subsets may then be correlated to the global gene expression screening before, during and after bone trauma in search for known and unknown genes of impact on bone regeneration. Observed differences should then be correlated to the different stages of bone regeneration as defined by the changes of cytokines and growth factors. Finally interactive cytomic, genomic and proteomic data analysis may reveal new biological phenomenons to be further studied in this human model.

Results and conclusions

Time dependent enumeration and multi parametric flow cytometry (MFC) analysis of mesenchymal progenitor subsets, gene expression profiling by microarray analyses of circulating cells and changes of circulating cytokine and growth factors in patients with traumatic hip fractures or planned hip replacements have resulted in the following important observations:

Non haematopoietic, $CD45^{neg}$MNCs, clustered in two compartments according to the temporal changes of the expression of surface membrane markers. Compartment 1 presented CD73,

CD105, CD144, CD133 and VEGFR2 while the complementary compartment 2 presented CD31, CD34, CD166 and CXCR4 on the cell surface.

Gene expression in circulating cells varied substantially from day 1 to 7 after trauma in 1463 genes in patients with hip fractures and in 1456 genes when comparing day 1 and 42 after trauma. Functional gene clusters indicated a stronger regenerative involvement from the circulating MNCs on day 7 than 42, due to higher EASE-scores of clusters involved in inflammation, cellular activity, cellular stress and tissue healing. The significant presence of selected specific benchmark genes also underlined the cellular involvement in bone regeneration, inflammation and neo-angiogenesis.

Elevated levels of YKL-40 and IL-6 were identified as a marker of tissue trauma and regeneration following bone fracture.

In conclusion this project has in a clinical model identified minor subpopulations of circulating potential progenitor cells, expressed genes and changes in cytokines during bone regeneration and the present hypothetic statement could not be rejected and gives room for more specific identification, isolation and characterization of mesenchymal progenitors and signal molecules with impact on fracture pathogenesis.

Future work and perspectives

These results are considered the first step in characterization of circulating subpopulations and genes expressed following bone fracture, which in combination with circulating cytokines and a growth factor during bone regeneration may improve our pathophysiological understanding of bone regeneration. Future work to characterize the model will involve 1) single cell sorting and gene analysis of subsets attempting to identify genetic markers to be used by Q-PCR assays to monitor bone healing and 2) protein analysis for known bone markers by ELISA and unknown biomarkers which support bone healing by global SELDI-FOF mass spectrometry The perspective is that this knowledge may be used in future clinical trials of regenerative medicine involving bone diseases.

INTRODUCTION

Bone Fracture

Bone fracture causes the formation of a fracture haematoma surrounding lacerated blood vessels, ischemic bone ends and adjacent soft tissue. The local tissue trauma and outflow of cytokines, results in a systemic inflammatory cascade of cells and molecules, which initiates the actual fracture healing or osteogenesis, consistent of intramembranous- and endochondral ossification (Figure 1A) (1;2). Intramembranous bone formation is an early and direct formation of osteoid bone matrix, originated from committed osteoprogenitor cells and uncommitted undifferentiated mesenchymal cells residing in the periosteum adjacent to the fracture site, resulting in an initial hard callus bridging the fracture gab with primary woven bone (3). The primary post traumatic vascular in-growth called neo-angiogenesis, is driven by the ischemic environment and signalling molecules in the fracture haematoma (4-7). Endochondral bone formation involves the recruitment, proliferation and differentiation of mesenchymal progenitors to form a cartilage anlage called a soft callus containing large proteins and proteoglycans that inhibit calcification until sufficiently degraded. The following enzymatically driven chondrocyte apoptosis and calcification precedes the infiltration of new blood vessels, which conduct the transport of undifferentiated mesenchymal progenitor cells with the ability to differentiate into osteoid producing osteoblasts, which turns this primary cartilage anlage into a hard bony callus that stabilizes the fracture (3;7). The calcified callus is visible on x-ray after 4-6 weeks (8). The temporal interplay between intramembranous- and endochondral bone formation creates spatial differences in the callus with calcified hard areas containing new woven bone in relation to the periosteum and soft cartilage containing areas adjacent to the actual fracture line (Figure 1B) (3;7). The regenerative process in fracture healing has several cellular and humeral similarities with the different stages of prenatal bone development, including preosteoblastic and mesenchymal progenitor cells, cytokines and growth factors (9-13). Remodelling of the hard bony callus consists of an opposing interaction between osteoclasts and osteoblasts, which conducts an elevated bone turnover for several months, and recreates the original architecture of the bone along stress lines established during active use of the limb (Figure 1C).

The phases of fracture healing can be monitored with clinical investigation, conventional x-ray, biochemical bone markers for bone formation and - removal (14-18). Bone regeneration

is also correlated to genes activated or silenced by detection of certain (messenger Ribo NucleicAcid) mRNAs in cells direct or indirect involved in bone formation, such as circulating or fixed immature preosteoblastic cells (19). It has been documented that the magnitude of the CRP and acute phase protein response is related to the mass of inflamed tissue and that serum IL-6 correlates closely with Injury severity score (20;21).

Definition of mesenchymal stem cells

The present paradigm on stem cells was founded by pioneers as Friedenstein, Owen and Caplan who comprehended the definition of stem cells as being plastic-adherent mononuclear cells with a fibroblast-like appearance, and ability to self-renewal while upholding a preserved potential to differentiate into multiple cell lineages (Figure 2) (22-24).

New technical methods of cell analysis regarding phenotype and function have widened the still growing field of stem cell research. The physical appearances of bone marrow derived progenitor cells is described by their small-, thin-, un-granular-, fibroblastic- or polygonal morphology after isolation and culturing (22;25-27). Further investigations have documented progenitor cell identity or immunophenotype based on the combination of surface markers and a cellular function based on gene expression, lineage commitment, in vitro- and in vivo culturing and transplantation assays of isolated MNC and MPC (26-31). The secretion of several haematopoietic and non-haematopoietic growth factors, cyto- and chemokines also clarifies the function and the heterogeneity of MPC-subpopulations (27;32). Human embryonic stem cells (ESC) can differentiate into all cell lineages in vivo and many types of tissue in vitro. (33;34). Ethical and practical issues impede the use of ESC as a therapeutic tool in treatment of degenerative, traumatic or congenital diseases (35;36).

Postnatal stem cell (PNSC) have smaller differentiating potential, but obvious immunological advances, are easy to produce and not encumbered by ethical considerations to the same extend as ESC (37). PNSC can divide many times without telomere shortening and without loosening their differentiation ability, which makes them a perfect material for future biological experiments and clinical treatment of diseases and traumas (25;30;38).

Immature primitive stem- or progenitor cells have been isolated from several kinds of tissue as muscle, skin, fat, synovium, periosteum, peripheral blood, bone marrow and even haemarthrosis (25;26;30;39-41). Bone marrow (BM) is an organ rich on putative PNSC. The PNSC-subpopulation of the bone marrow located mononuclear cell cluster (BMMNC) can be

divided in haematopoietic and non-haematopoietic progenitor cell populations based on their differentiation potential and presence of surface markers (25;30;38;42). Bone marrow could be considered as a stock of non-circulating MPCs, because of relative high number of MPCs compared to peripheral blood and the close relation to the circulation, which makes secretion of the MPCs easier (26;43;44). The number of MPCs in peripheral blood is very low at normal homeostatic conditions, which makes the isolation and further study of this cell population quite difficult in healthy persons. Several research groups have unsuccessfully tried to isolate MPC from peripheral blood, primarily due to low numbers of cells, but also due to differences in isolation and culturing methods (44-47). If the function of these cells is to participate in regeneration, they might only be measurable with the current techniques after a certain trauma, which conducts tissue hypoxia or inflammation, which again can activate the bone marrow derived MPCs and increase the circulating pool of these cells (48).

The nomenclature

The nomenclature is not unanimously on non-haematopoietic stem cells and comprehend terms as Colony Forming Units Fibroblasts (CFU-Fs), skeletal stem cells (SSC), mesenchymal stem cells (MSC), marrow stromal cell (MSC), multi potent mononuclear stem cells, mesodermal stem cells, mesodermal progenitor cells (MPC), mesenchymal progenitor cell (MPC) or multipotent adult progenitor cells (MAPC) (30)(30)(22;25;26;30;40;49;50).

In this thesis the generic term mesenchymal progenitor cell (MPC) will be used, which covers a population of committed progenitor cells with ability to differentiate into more than one specialized cell type of mesenchymal origin. The MPC has been isolated from bone marrow and peripheral blood. As an initiative to standardize the nomenclature concerning stem cells, The international Society for Cellular Therapy (ISCT) defines a multi potent mesenchymal stromal cell as a fibroblast-like plastic-adherent cell in spite of tissue of origin (51).

Surface membrane CD markers

As with the nomenclature there is no consensus on which surface markers that determines the exact stem- or progenitor cell phenotype, but some surface markers are considered to be characteristic for this non-haematopoietic progenitor cell population.

It is widely accepted that BMMSC are CD14, CD34, CD45 and CD133 negative due their non-haematopoietic abilities and positive for STRO-1, CD73, CD90, CD105, CD106, CD117 and

CD166 or at least a combination of these. Due to differences in isolation and culturing methods, it is critical to use multiple surface markers to verify the identity of the investigated cell populations (26;28-30).

Increasing knowledge of progenitor cell phenotypes could affect the efficiency of MPC characterisation, isolation and use of MPC in future therapeutic perspectives (29;44). A resent study by Dennis and co-workers documented that the presence of the surface markers CD90, CD105 and CD166, had an important impact on the osteogenic potential of that cell population (28). The chemokine receptor CXCR4 has been associated with circulating progenitor cell activity, because cells with this surface marker are migrating towards hypoxic and traumatized tissue secreting stromal cell derived factor 1 (SDF-1) (52-54).

Gene expression analysis

Microarray analysis of gene expression quantifies the expression of numerous genes, which enables this technique to determine the overall genetic response in a cell population, in one single test. The genes can be clustered according to their level of expression and their function, which reflects the cellular response on a physiological event. The importance for the cellular function of each gene cluster is quantified by EASE-scores (enrichment-score). An EASE-score of 1.3 is equivalent of non-log scale 0.05, meaning that attention should be given towards gene-clusters with EASE-scores >1.3. The gene–clusters are a combination of sub-clusters, of which the probability of importance is quantified by a p-value. It is further possible to screen for specific genes with known functions, so called benchmark genes, between numerous of unknown genes. Presence of benchmark is a very strong indication of the cellular function.

The material applied in microarray analysis is mRNA. Even though not all of the mRNA will be translated into proteins in the ribosomes, the quantitative analysis of the mRNA gives an estimate of the overall gene expression and thereby the most important function of the cell population. In the laboratory the mRNA is extracted from the cell, a double stranded cDNA and subsequently a single cRNA strand is produced, which is fragmentized, labelled and in a hybridization solution and incubated over night on the gene-chip, where the cRNA fragments binds up to 11 of the several hundred thousand probes on each gene chip. The binding of the labelled cRNA-sequences to the gene chip are quantified, by comparing the fluorescence intensity of the bound cRNA with some control probes on the same chip. The chips are laser

scanned and the intensities of the numerous probes are background corrected and normalized. In this rapport we evaluated the temporal effect of bone regeneration on gene expression. Genes that are significantly up- or down regulated, with respect to a chosen false discovery rate (FDR) are included in the analysis. A FDR is used to avoid false discoveries of significantly expressed genes, which will occur, when analysing several hundred thousand probes in one test (55-58). A chosen part of the transcriptomes, called a gene list, can be applied to gene ontology analysis (GO-analysis) or The Database for Annotation, Visualization and Integrated Discovery (DAVID-analysis), which combines knowledge of the molecular, cellular or biological functions of the single gene with the expression level, and thereby results in a network or cluster of related genes based on their function and expression level (59).

Colony forming efficiency (clonogenicity)

A central characteristic of progenitor cell is the ability to form adherent colonies of fibroblastic cells, when seeded in plastic dishes, hence the name CFU-F (colony forming unit fibroblast-like cells). The concentration of progenitors in cell populations of different origins is quantitatively evaluated by their clonogenicity or colony forming efficiency (CFE). The CFE describes the number of colonies that grows out when $1x10^6$ mononuclear cells (MNC) are seeded in a Petri-dish.

The CFE in peripheral blood (PB) is $1.2-13/10^6$ MNC in rabbits and $0.5-5/10^6$ MNC in humans under normal conditions, which is considerable lower than in human bone marrow (BM) were the CFE is $20-35/10^6$ MNC (26;43). The CFE rises during pathological conditions, as in haemarthrosis from distorted knees, where the CFE is $20-35/10^6$ MNC or in synovium from arthritic knees, where the CFE is as high as 1.25-8% of the plated MNC. The number of CFE in synovium from arthritic knees exceeds the CFE in BM by far, which must be a result of cellular homing in the synovium (29;39;41;43).

Even though there is a 20-30 fold difference in the CFE between PB and BM, the MPC-colonies derived from culturing cells from PB and BM, have an equally large osteogenic potential, when qualitatively measured as “regeneration of bone traumas by x-ray” and quantitatively evaluated by histochemestry or BMD-measurement with CT-scans (26;39;43).

Multi potent adult progenitor cells

This project is based on the detection of a population originally designated Multi potent adult progenitor cells (MAPC) by Verfaillie and co-workers (38). MAPC was purified from the non-haematopoietic fraction of BMMNC and characterized by differentiation abilities, surface antigens, gene analysis by RT-PCR, immunohistochemistry and immunofluorescence, which separate MAPC from MSC, though both populations are derived from BMMNC (38;40). Interestingly it has been documented that MAPC can differentiate into cells of both endo- and mesodermal germ layers (37;38). These discoveries categorize MAPC as being developmental or phylogenetically more primitive than progenitor cells with differentiation potential restricted to only mesenchymal tissues (Figure 2). In a resent study Price and co-workers identifies and isolates MAPC from peripheral blood MNC and has designated this cell population as peripheral blood derived multi potent adult progenitor cells (PBD-MAPC). The PBD-MAPC fulfil the stem cell properties as being self-renewing and able to differentiate, in this case without a feeder layer which makes these cells interesting for future clinical treatment (60).
Beside the MAPC population, Minguell describes a population of uncommitted bone marrow derived mesenchymal progenitor cells with the ability to differentiate into a neural cell line, which is of ectodermal origin (27).

Research status on circulating mesenchymal progenitor cells

Animal studies have documented that pro-inflammatory cytokines, chemokines and growth factors released from traumatized tissue activates a population of primitive multi potent MPC in the bone marrow (7;13;31). MPC are then secreted into- and distributed by the circulation throughout the organism. When homed at the fracture site, the local micro environment attenuates the differentiation of the MPCs in an osteo- or angiogenic direction to replace the traumatized tissue, regain vascularisation and the lost function. Some studies further arguments that MPC mediates the microenvironment through secretion of specific peptides (26;32;39;42;43;48;61).
The number of MPC needed to regenerate tissue in orthopaedic traumas is unknown, but resent studies has documented the possibility to raise the number of cells with regenerative potential and stimulate bone and cartilage regeneration (62-66). Some state that the in vitro expansion of MPC to sufficient numbers typically takes two to three weeks (28).

Some groups use the original term MPC and try to isolate the plastic adherent cells from peripheral blood (26;39;67), while others study MPC in the non-adherent compartment of circulating mononuclear cells (44;68).

There are substantial similarities between PBMPC and BMMSC indicating that they are strongly related and that PBMPC is a secreted subpopulation from the BMMSC cluster. Both populations share some important features as low granularity, being mononuclear, important surface markers, gene expression, differential potential within and beyond mesenchymal lineages and ability to adhere to plastic (69-71).

Circulating MPC can engraft in bone, muscle, brain, lung, heart, liver, gastrointestinal tract and haematopoietic system when transplanted (72-74).

Future use of stem cells in optimising bone healing could be important in situations with poor bone healing abilities, reconstruction of larger bone defects due to trauma or surgery, osteoporosis, congenital bone defects and faster and better implantation of prosthesis (75;76). Some studies have shown great and useful regenerative potential in application of bone marrow derived stem cells in traumatized bone defects, though still only in animal models (62;64;65;76). The necessity of knowing the exact identity of the reinserted cells still makes the use of stem cells in clinical treatment difficult.

YKL-40, IL-6 and CRP documenting inflammation and bone regeneration

To investigate the humeral response on bone trauma, we studied the inflammatory cytokines IL-6 and CRP and the growth factor YKL-40. IL-6 is related to inflammation as well as endochondral ossification and remodelling (2;3;7;13;77). CRP is related to unspecific quantification of tissue trauma, treatment and postoperatively complications as deep wound infection or bronchopneumonia (78-81). The YKL-40 level is among other things related to cartilage trauma in arthritic joints and to the initial repair response (82-88). Stoffel and co-workers showed that serum YKL-40 increases rapidly after a malleolar or tibia fracture, followed by a decrease shortly after osteosynthesis.

RESULTS

The described aims were studied by an analytic strategy of circulating cells and molecules in plasma from patients with hip fracture, who healed through posttraumatic bone regeneration and patients with planned hip replacements without any posttraumatic bone regeneration, in an attempt to give answers to the questions below related to bone regeneration:

Is it possible to identify and enumerate predefined subpopulations of circulating non haematopoietic progenitors by multiparametric flow cytometry?

Do screening of global gene expression in mononuclear cells from patients with hip fractures and age matched controls document any posttraumatic genetic difference between these groups, and uncover known or new genes or genetic clusters involved in pathogenesis of bone regeneration?

Are selected circulating cytokines and growth factors quantified by ELISA involved?

The answers are given in the result section below. The planned quantitative analysis of a range of selected cytokines and growth factors was not performed at the end of the PhD stipend but will be performed in the near future.

The study cohorts, model and Biobank

Two cohorts with a total of 50 patients with ankle- or hip fracture or planned hip replacement and 17 normal age-matched persons without any known musculoskeletal morbidity were included. Interviews, demographical and clinical data from their medical journals were used to in- or exclude the patients as shown in Table 1. All participants had blood samples taken before and from day 1-84 after bone trauma (Figure 3). Cohort 2 was included because the flow cytometry analyses on thawed cells from cohort 1 were inconsistent and because the serum values of YKL-40, IL-6 and CRP rose significantly within the initial 24 hours after bone trauma. Patients with ankle fractures were not included in cohort 2, because of their low posttraumatic response on circulating MNCs in cohort 1. Gene expression analysis were only done on material from patients with hip fractures from cohort 2 and contemporary healthy persons, because these patients showed a posttraumatic cellular response in cohort 1 and because the flow cytometry analysis on these patients indicated a posttraumatic role for circulating non-haematopoietic progenitor cells during bone regeneration, which was not seen in patients with hip prosthesis. An overview of the applied methods of analyses summed up that all 50 patients in cohort 1 and 2, together with the 17 healthy controls had quantified

their circulating MNCs, YKL-40, IL-6 and CRP, but the flow cytometry analysis were solely done on isolated un-frozen MNCs from cohort 2, as were the quantifications of YKL-40, IL- and CRP within the initial 24 hours. The gene expression analyses was only done on material collected before-, day 7 and 42 after surgery from 6 patients with hip prosthesis and material collected day 1, 7 and 42 after fracture from 6 patients with hip fractures, all patients were from cohort 2. We focused the further statistical gene expression analysis on data from patients with hip fractures and healthy controls from cohort 2, because of our interest in the posttraumatic genetic response in circulating MNCs during bone regeneration. We found it reasonable to believe that such a genetic response could be determined in this material because of the documented cellular involvement in posttraumatic bone regeneration in these patients. We found it reasonable to use the healthy controls as estimated cellular and genetic baseline material for the contemporary patients with hip fractures. The results of the gene expression analyses is focused on three parameters, which consists of the number of genes that have higher expression levels at day 7 and 42 after fracture compared to day 1, the presence and EASE-score of functional genetic clusters in the gene list at day 7 and 42 after fracture and to determine the presence of genes with known relation to bone regeneration, so called benchmark genes, in the genes which had higher levels of expression in the fracture patients than in the healthy controls.

MNCs, serum and plasma from the 50 included patients and the 17 healthy controls was isolated from the collected blood and stored in a research Biobank for later analyses. $1x10^7$ of the isolated MNCs from patients in cohort 2 and healthy controls were applied to the flow cytometer before freezing, while some MNCs from the same samples day were frozen fro later analysis. The cells are kept at -80°C, while plasma is kept at -20°C, because these temperatures were considered sufficient low for later thawing and analyses of the material. A more detailed description of analysis is present in Material and Method section of the supplementary material and the included manuscripts.

Enumeration of circulating WBC, MNC and platelets

The number of circulating WBCs changed during bone regeneration in all patients in cohort 1 (P<0.0001) (Figure 4A), but only in patients with planned hip replacements in cohort 2 (P=0.009) (Figure 4B). Platelets changed significantly in traumatic and surgical bone traumas

in cohort 1 and cohort 2 (P<0.0001) (Figure 4). MNCs changed in traumatic and surgical bone traumas in cohort 1 (P<0.0001) (Figure 4A) and cohort 2 (P=0.006) (Figure 4B).

The number of circulating WBC, platelet and MNCs in patients with hip fractures in cohort 2 normalized at day 21-28, which is a considerable delay compared to patients with planned hip replacements that normalized day 3-7. The later normalization of circulating WBC, platelet and MNCs indicated a longer and more intensified use of these cells during regeneration of traumatic than surgical bone traumas (Figure 4B).

Changes in WBC, MNC and platelets corresponded to the magnitude of traumatized bone in cohort 1, documented by a higher posttraumatic response in patients with hip fractures and patients with planned hip replacements, than in patients with ankle fractures (MNC: P=0.0006, WBC: P=0.04, platelets: P=0.02) (Figure 4A).

Quantitation and phenotyping of circulating MPC

It was possible to reach our predefined aim on identifying and quantifying circulating subpopulations of non haematopoietic progenitor cells by combining MFC-data and MNC-enumeration. The changing concentrations of the surface markers on the MNCs divided these in two clusters (Figure 5).

Cluster 1 changed in accordance with the $CD45^{neg}$MNC compartment and consisted of $CD45^{neg}$MNCs with presence of CD144, CD105, CD73, CD133 and VEGFR2.

Cluster 2 changed differently than the $CD45^{neg}$MNC compartment and consisted of $CD45^{neg}$MNCs with presence of CD31, CD34, CD166 and CXCR4. The strong internal correlation, between the changes of the surface markers on the cells within each cluster, underlined the cluster-integrity.

Trauma related changes of cells in cluster 1 and cluster 2

Cluster 1 peaked at day 3-7 in the fracture-patients and at day 7 or 14 in patients with panned hip replacements, followed by a uniform plateau from day 21 to 84 in all the patients (Figure 6A-D).

The cells in cluster 2 changed concentration depending on the type of bone trauma in contrast to cells in cluster 1.

The general level of positive surface markers on the cells in cluster 2 were higher in patients with planned hip replacements than in patients with hip fractures throughout the study period (P=0.049, P=0.002, P=0.02 and P=0.047) (Figure 7A-D).

Cells in cluster 2 showed a unique pattern in patients with hip fractures with a decline from day 3 and 7 to day 14 (CD31:P=0.01, CD34:P<0.0001, CD166: P=0.02 and CXCR-4:P=0.02). The concentration of $CD31^{pos}$, $CD166^{pos}$, CXCR-4^{pos}, $CD45^{neg}$MNCs rose day 14 to a plateau from day 21 to 84 in patients with hip fractures. $CD34^{pos}$, $CD45^{neg}$MNCs declined from day 42 in patients with hip fractures (Figure 7D).

Cells in cluster 2 did not change concentration until day 21 in patients with planned hip replacements. From day 21 $CD31^{pos}$, $CD166^{pos}$, CXCR-4^{pos}, $CD45^{neg}$MNCs rose and peaked uniformly at day 42 followed by a significant decline to day 84 (CD31:P=0.006, CD166:P=0.002, CXCR-4:P=0.02) (Figure 7A-C). $CD34^{pos}$, $CD45^{neg}$MNCs did not change significantly during the study period in patients with planned hip replacements (Figure 7D).

Gene expression screening by microarray analysis

During the data generation Affymetrix platform turned the 53 (6 patients with hip prosthesis before, at day 7 and 42 after surgery, 6 patients with hip fractures at day 1, 7 and 42 days after fracture and 17 healthy controls) microarray slides into cel-files ready for data analysis (89).

All subsequent statistical analyses were performed using Bioconductor packages (90) which are add-on modules for the statistical package R, (R Development Core Team, 2004).

As we experienced that the two treatment groups have considerably variations in their gene-expression patterns over time, we chose to normalise and analyse the three data-sets separately. Only in this way were we sure to maintain interesting time variations in each group during normalization. A batch effect across the treatment groups was noticed. We adjusted for this effect by centralising each group towards the grand mean of all probes.

The quality of the raw data was inspected by RNA-degradation plots and histograms for each patient by routines from the Bioconductor package arrayQualityMetrics (91). No slides showed poor quality (data not shown).

Background correction and normalisation was carried out by the Robust Multichip Average procedure (RMA) found in the Bioconductor package affy (92). We made an unspecific prefiltering for the hip fracture and hip prosthesis groups with nsFilter from the Bioconductor

package genefilter. This procedure considerably brought down the number of probes. No filtering of the healthy control group was performed as this group acts as control in later comparisons.

For each treatment group a linear mixed effects model was fitted to the logarithm of the gene expressions, with patients modelled as a random effect to take into account the inter-person correlation. Time was modelled as a fixed effect. The bioconductor package Limma (93) was used to fit the model. Differentially expressed genes between time points were identified by consideration of moderated *t*-tests for relevant contrasts. The moderated *t*-test is based on empirical Bayes analysis, and is equivalent to shrinkage of estimated sample variances towards a pooled estimate, resulting in more stable inference when the number of microarray experiments is small. Due to the high number of false positives introduced because of multiple testing, we used the Benjamini-Hochberg (BH) *q*-values to control or estimate the false discovery rate. The meaning of "BH" q-values is as follows. If all genes with q-value below a threshold, say 0.005, are selected as differentially expressed, then the expected proportion of false discoveries in the selected group is controlled to be less than the threshold value, in this case 0.5%.

Comparisons between the hip fractures and hip prosthesis groups versus the healthy control group were performed by heatmaps. We used the heamap.2 function from the R-package gplots. The hierarchical cluster analysis indicated in these heatmaps was based on the R-function hclust using average as the agglomeration method and Euclidian distance as dissimilarity measure.

Gene expression in circulating MNCs from 6 patients with hip fractures from cohort 2 on day 1, 7 and 42 after fracture and 17 healthy controls were quantified by microarray analysis. The gene expression analysis is as mentioned focused on three parameters to determine the genetic posttraumatic response.

With a false discovery rate (FDR) at 0.5%, 1462 genes changed expressions level from day 1 to 7 in patients with hip fractures. The number of differently expressed genes at day 42 compared to day 1 in patients with hip fractures were even 1456. By using the healthy controls as estimated normal persons, we can illustrate, as expected, the gene expression in these patients changes violently within the first 24 hours after the fracture and then normalizes towards the pre-fracture homeostatic equivalence during 42 days of bone regeneration (Figure 8). We investigated the presence of functional gene clusters among

genes with a higher expression level day 7 than day 1 (HF 7 vs. 1) and day 42 than day 1 (HF 42 vs. 1), by using the webbased gene enrichment tool DAVID, version 2008.html. Analysis of HF 7 vs. 1 resulted in the formation of several gene-clusters, which were involved in inflammation, cellular activity and cellular stress (Table 3). A similar analysis of HF 42 vs. 1 showed some of the same gene-key-clusters, but these had lower EASE-scores and the sub-clusters had lower p-values (Table 4). This difference in gene expression and functional gene clusters documented the involvement of circulating MNCs in bone regeneration on day 7 and 42, but with a stronger involvement in inflammation, cellular activity and cellular stress due to tissue stress on day 7 than on day 42. The key-cluster "Tissue healing" was not present at day 42, as it was on day 7 (Table 3 and 4).

To determine actual presence of genes coding for specific cytokines and growth factors, which are known to be involved in bone regeneration, inflammation and neo-angiogenesis, analysed the presence and p-values of so called benchmark genes these genes in the circulating MNCs during bone regeneration on day 7 and 42. Genes coding for VEGF (Vascular Endothelial Growth Factor), OC (Osteo Calcin), IL-6 (Interleukin 6), CXCR4 (Chemokine Receptor 4) and TGF-β (Transforming growth factor beta) were significantly expressed on day 7 and 42, except for TGFB1, which codes for TGF-β, that were only significantly expressed at day 7 (Table 5). The presence and p-values of benchmark genes in circulating MNCs together with the heatmap that showed the temporal changes of posttraumatic gene expression at day 1, 7 and 42 and in healthy controls strongly indicates a cellular involvement in bone regeneration that changed gene expression towards a normal homeostatic cellular activity during the first 42 days after hip fracture.

Circulating biomarkers after bone trauma

YKL-40 increased post traumatic in patients with hip fractures ($P=0.0001$) and patients with planned hip replacements ($P<0.0001$) in cohort 2 (Figure 9). As mentioned in the summery the YKL-40 level was higher day 14-42 in patients regenerating from fracture than after planned hip replacements in both cohort 1 ($P=0.04$) and cohort 2 ($P=0.005$) (Figure 9).

Serum IL-6 rose from trauma to day 1 ($P<0.0001$) and declined in a similar pattern in all three patient groups in cohort 1, indicating that IL-6 quantifies the same posttraumatic inflammatory process in the three patient groups (Figure 9)

Serum CRP rose ($P<0.0001$) in all patients in cohort 1. Changes in s-CRP is considerable in patients with hip fractures ($P<0.0001$) and patients with planned hip replacements ($P<0.0001$) in cohort 2 (Figure 9). As for YKL-40 and IL-6 the post traumatic CRP levels correlated to the magnitude of the bone trauma, documented by different levels in traumatic or planned hip traumas and ankle fractures (YKL-40:$P=0.0004$, IL-6:$P<0.0001$, CRP:$P=0.0021$) (Figure 9).

YKL-40 and $CD105^{pos}$, $CD45^{neg}$MNCs ($r=0.26$, $P=0.01$) and $CD144^{pos}$, $CD45^{neg}$MNCs ($r=0.27$, $P=0.01$) were positively correlated, but only in patients with ongoing endochondral ossification after hip fractures (Figure 10). This positive correlation is a novel finding, which strongly underlines the pivotal involvement of these cells and the growth factor YKL-40 during a very important post-inflammatory period of bone regeneration.

SPECIFIC SUMMARY

The results from the study of circulating mononuclear cells and specific cytokines and a growth factor involved in post traumatic bone regeneration is summarized below:

A post traumatic cellular response in the non haematopoietic CD45neg compartment was present and defined two subpopulations by cluster analysis.

Cluster 1 consistent of CD45^{neg}MNCs with presence of CD73, CD105, CD144, CD133 and VEGFR2 on the cell surface.

Cluster 2 consistent of CD45^{neg}MNCs with presence of CD31, CD34, CD166 and CXCR4 on the cell surface.

Cells in cluster 2, in contrast to cluster 1, changed concentrations differently than the CD45^{neg}MNC population, had quite high concentrations of surface markers, presence of CXCR4 that is related to circulating progenitor cells and changed concentration according to type of bone trauma.

Global gene expression were related to the type of bone trauma, documented by substantial changes in number of expressed genes in patients with hip fractures in contrast to patients with hip replacements.

Patients with hip replacements have a more stable genetic expression over time, than patients with hip fractures, which underline the relation between the type of bone trauma and posttraumatic changes in gene expression.

Functional gene analysis showed genetic clusters involved in bone regeneration on day 7 and 42, but with a stronger involvement in actual trauma response on day 7 than 42.

Bone trauma conducted a rise in YKL-40, IL-6 and CRP.

Traumatic bone trauma conducted a rise in YKL-40, IL-6 and CRP before corrective surgery.

Correlation between the magnitude of bone trauma and rise of YKL-40, IL-6 and CRP.

IL-6 rose in contrast to CRP during bone regeneration between day 14 and day 42.

YKL-40 was elevated during bone regeneration between day 14 and day 42 and normalized in patients without co-morbidity at 12 months.

DISCUSSION

In the pursue of the identity and quantity of circulating mesenchymal progenitor cells involved in posttraumatic bone regeneration, we set up a clinical model to investigate the cellular and humeral response on bone trauma and applied it on 50 model patients with ankle- or hip fractures or planned hip replacements and 17 contemporary healthy controls. The studied material consisted of peripheral blood derived MNCs and cytokines from serum and plasma. The manipulation of the peripheral blood derived MNCs was minimized and consisted of isolation, enumeration, flow cytometry analysis on un-frozen cells and gene expression analysis on thawed MNCs. The serum and plasma was collected from the blood samples and frozen for later analysis.

We quantified the circulating MNCs, because the mesenchymal progenitor cells are believed to be a subpopulation of this heterogeneous cellular compartment, but also because we wanted to investigate the cellular inflammatory response on bone trauma, because this could be involved in bone regeneration. Our model enabled us to determine a posttraumatic cellular response, even though the result was rather unspecific due to the heterogeneity of the MNC-compartment. This simple analysis further documented a correlation between the magnitude and type of bone trauma and the number of circulating MNCs.

To identify and quantify the presence of circulating mesenchymal progenitor cells we determined the percentage of specific surface markers with multi parametric flow cytometry on un-frozen MNCs and multiplied this percentage with the number of MNCs from the original blood sample, as mentioned above. Because mesenchymal progenitor cells are believed to belong to the compartment of non-haematopoietic progenitor cells, our model determined the concentration of non-haematopoietic progenitor cells within the compartment of circulating MNCs by determining the concentration of MNCs that were $CD45^{neg}$. The methodological problem of defining a population by determining what it is not was encumbered by the presence of surface markers on the $CD45^{neg}$MNC-compartment. The flow cytometry analysis resulted in two rather homogeneous clusters of cells defined by the presence of surface markers on $CD45^{neg}$MNCs, which changed concentration in a similar pattern during bone regeneration. Cluster 1 consisted of $CD45^{neg}$,$CD73^{pos}$,$CD105^{pos}$,$CD133^{pos}$,$CD144^{pos}$ and $VEGFR2^{pos}$MNCs. Cluster 2 consisted of $CD45^{neg}$,$CD31^{pos}$,$CD34^{pos}$,$CD166^{pos}$ and $CXCR^{pos}$MNCs. Based on the phenotypes of these clusters, cluster 1 could consist of mesenchymal progenitor cells and cluster 2 could consist of endothelial progenitor cells, which both are important for

posttraumatic neo-angiogenesis and actual bone replacement. When combining the flow cytometric data with the clinical and demographical data on the model patients, our model determines interesting temporal and trauma-related differences between the two clusters of progenitor cells and the types of traumas, which arguments for the contribution of the different cell clusters to bone regeneration. We believe that the method is usable and strong because all the patients are followed during the 12 weeks of bone regeneration and are therefore their own control persons, documenting the changing concentration of certain subpopulation of progenitor cells during different stages of bone regeneration. The flow cytometry analysis of the circulating MNCs documented not only a trauma and magnitude specific cellular response, which was even more specific than the MNC counting, but also that these analyses differed depending on mode of regeneration.

The final parameter of the cellular investigations on circulating MNCs consisted of analyses of the gene expression in the peripheral blood derived MNCs. The gene expression analyses documented an immediate genetic response in patients with hip fractures, which was quite notable documented by the number of genes that changed expression level after fracture. The qualitative part of the gene expression analyses showed a genetic involvement in the posttraumatic bone regenerative response by presence of clusters with strong relations to tissue trauma, inflammation and cellular activity. The presence of the significantly expressed benchmark genes in the gene lists also indicated a strong relation of the genetic response in these cells to the bone trauma.

We believe that the humeral part of our model supported the cellular part effectively by documenting a significant rise during inflammation in YKL-40, IL-6 and CRP, which correlated to the magnitude of traumatized bone, but also during the actual bone regeneration, with a significant difference in YKL-40 levels in patients according to the type of bone trauma. This was strongly documented by a positive correlation between a specific cluster of circulating mesenchymal progenitor cells and the posttraumatic levels of YKL-40, which was only seen in patients with hip fractures.

We believe it is recommendable to use this thorough clinical model to evaluate the posttraumatic cellular response on bone trauma with or without bone regeneration, because it documents the phenotype of the involved cells, quantifies the changes of MNCs, CD45^{neg}MNCs and the clusters of mesenchymal progenitor cells defined by presence of several surface markers during bone regeneration and shows a genetic involvement in bone

regeneration. Further more are the cellular data supported by inflammatory and bone regenerative cytokines and a growth factor.

Based on this thesis, several scientific problems arise, which must be investigated in future studies. The two main questions that will need to be addressed in future studies are the exact cytokine and growth factor response on bone trauma, documented by a more specific analysis of the molecules in serum and plasma during bone regeneration and the specific genetic expression in the two homogeneous clusters of mesenchymal progenitor cells.

Knowledge of- and ability to quantify normal bone regeneration is important for future quantification and optimisation of fracture healing. Quantification of YKL-40 during regeneration of surgical or traumatic bone traumas could result in the discovery of an early and effective quantitative prognostic marker of bone formation.

We believe that our hypothesis was tested sufficiently and that the arguments above supports the existence of circulating clusters of mesenchymal progenitor cells in measurable numbers in patients during ongoing bone regeneration.

FIGURE AND TABLE LEGENDS

Figure 1

A schematic overview of posttraumatic bone regeneration. Formation of a fracture haematoma surrounding lacerated blood vessels, ischemic bone ends and adjacent soft tissue marks the inflammatory phase (1A). The temporal interplay between intramembranous- and endochondral bone formation creates spatial differences in the callus with calcified hard areas containing new woven bone in relation to the periosteum and soft cartilage containing areas adjacent to the actual fracture line (1B). Remodelling of the hard bony callus consists of an opposing interaction between osteoclasts and osteoblasts, this is called the remodelling phase and continues for months (1C).

Figure 2

A simplified model of the stages of differentiation of mesenchymal progenitor cells, from completely uncommitted self renewing undifferentiated mesenchymal stem cell (MSC), over multipotent adult progenitor cells (MAPC) with preserved ability to self renew, but a higher degree of commitment to the mesenchymal linage, through the fully committed, but still pluripotent mesenchymal progenitor cell (MPC) ending at the tissue specific end stage cells. The cell number rises with a falling degree of stemness.

Figure 3

Time line of the blood samples. Cohort 1 include AF (ankle fractures), HF (hip fractures) and THR (total hip replacements). Cohort 2 includes HF and THR, shown on two different time lines due to the difference in times of drafting the initial blood samples. BS=Before Surgery, blood samples were collected within 1 week before surgery).

Figure 4

Quantification of circulating leucocytes, mononuclear cells and platelets in cohort 1 as shown in 4A and cohort 2 as shown in 4B.

Healthy controls had an average age of 71 years (63-81) and a mean number of circulating leucocytes of 6.53 x 10^9/L (4.1 x 10^9/L–8.8 x 10^9/L), MNC of 2.53 x 10^9/L (1.19 x 10^9/L 3.57 x 10^9/L).

Figure 5

The CD45^{neg}MNC clusters in two subpopulations according to the changing intensities of the specific surface markers during bone regeneration. The dendrogram illustrates the division of the surface markers in two clusters designated cluster 1 and cluster 2. The numbers in the squares are the correlation coefficients, which are also shown in the colour key.

Figure 6

Changes in CD45^{neg}MNC (6A), CD73^{pos}-CD45^{neg}MNC (6B), CD105 ^{pos}-CD45^{neg}MNC (6C), CD144 ^{pos}-CD45^{neg}MNC (6D) (Cluster 1) during bone regeneration.

Figure 7

Changes in CD31^{pos}-CD45^{neg}MNC (7A), CD34 ^{pos}-CD45^{neg}MNC (7B), CD166 ^{pos}-CD45^{neg}MNC (7C) and CXCR4^{pos}-CD45^{neg}MNC (7D) (Cluster 2) during bone regeneration.

Figure 8

The heatmap shows the standardised values of the 1000 most time-varying genes. Red are row-wise higher values and blue are row-wise lower values. The genes are row-wise sorted by a cluster algorithm according to their similarity with other genes. Likewise are the samples column-wise sorted by a cluster algorithm according to their similarity with other samples. Red are the samples at time 1, blue the samples at time 3 and green the samples at time 42. The yellow samples are the healthy controls.

Figure 9

Changes in YKL-40, IL-6 and CRP during bone regeneration

9 A,C,E are YKL-40, IL-6. CRP from cohort 1

9 B,D,F are YKL-40, IL-6. CRP from cohort 2

Dotted lines mark upper boundaries of normal levels.

The average levels of YKL-40 in patients with hip fractures from cohort 2 were 116 µg/L and 68 µg/L in patients with hip replacements. These patients had an average level of IL-6 at 3.35 µg/L. The average level of YKL-40 in patients from cohort 1 is 83µg/L. A CRP value <10 mg/L are considered as non-pathological.

Figure 10

The positive correlation between YKL-40 and circulating CD105 ^{pos}-CD45^{neg}MNC (10A) and CD144 ^{pos}-CD45^{neg}MNC (10B). Such a correlation was only seen in patients with traumatic hip fractures.

Table 1

Demographic and clinical data on patients and healthy controls, which completed the study period.

Table 2

The surface markers analyzed on the isolated MNCs. The panel of surface markers consists of antibodies with known relation to endothelial-, haematopoietic and mesenchymal progenitor cells.

Table 3

Key- and sub-clusters of genes with possible relation to the ongoing posttraumatic inflammation or bone regeneration. The genes are differentially expressed at day 1 and day 7 in patients with hip fractures.

Table 4

Key- and sub-clusters of genes with possible relation to the ongoing posttraumatic inflammation or bone regeneration. The genes are differentially expressed at day 1 and day 42 in patients with hip fractures.

Table 5

Analyses of Benchmark-genes involved in inflammation, neo-angiogenesis and bone formation. The expression of such benchmark-genes documents the relation of a specific cell population to a specific physiological process.

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Table 1

Bone trauma	No. of pt. in./ex./ completed	Age/ years	Sex	Co-morbidity		Complications affecting bone regeneration	Total Blood samples
				total	diagnoses		
AF	14/1/13	47(22-68)	7M 6F	2/13	2 RD	3/13	8
HF (1)	14/4/10	77(60-89)	5M 5F	5/10	1 GI, ND, t.CP 1 RD,OP, t.ED 1 OP 1 OP, t.CP,ND 1 AS	6/10	8
THR (1)	9/9	70(44-84)	2M 7F	9/9 OA 3/9 nOA	1HT 1OP,RD,t.CP 1RD	1/9	8
HF (2)	13/5/8	85(75-90)	8F	7/8	1 DE 1 t.CP 1 OP 1 DE 1 OP,t.ED,RD 1 HP,RD 1 ND,DE	2/8	10
THR (2)	12/2/10	68(50-80)	2M 8F	10/10 OA 6/10 nOA	1 t.CP 1 t.CP 1 t.CP 1 HP,t.ED 1 HP, t.ED 1 HP	1/10	9

(1) Study 1. (2) Study 2. AF – ankle fracture, HF – hip fracture, THR – Total hip replacement. OA – osteo arthritis, t.CP – treated cardiovascular illness, HC – high cholesterol, t.ED – treated endocrine disease, DE – dementia, OP – osteo porosis, RD – rheumatoid disease, HP – high blood pressure, nOA – non osteo arthritic disease, GI – Gastro intestinal disease, ND – Neurological disease, AS – Artery sclerosis,

Table 2

Associated cell population	Cluster of differentiation	Antigen	Conjugation
Leukocytic common antigen	CD45	LCA	PerCP
Haematopoietic progenitor cell	CD34	Glycoprotein 105-120	APC
Haematopoietic progenitor cell	CD133	AC133	PE
Mesenchymal progenitor cell	CD73	Ecto-5'-nucleotidase	PE
Mesenchymal progenitor cell	CD105	Endolgin	FITC
Mesenchymal progenitor cell	CD166	ALCAM	PE
Endothelial cells	CD31	PECAM-1	FITC
Endothelial progenitor cell	CD144	VE-cadherin	FITC
	VEGFR-2	VEGF 2	PE
Injured and hypoxic tissue secreting SDF-1	CXCR4 (CD184)	CXCR4	PE
Negative control	IgG1(Mouse)		FITC, PE, PerCP, APC

Table 3

Key cluster	EASE-score	Sub-cluster	P-value (Benjamini)
Transcription factor binding	5.7	Transcription factor binding	4.4E-6
		Trascription cofactor activity	7.5E-4
Apoptosis	4.86	Regulation of apoptosis	1.7E-5
		Cell development	1.6E-2
		Cell differentiation	3.3E-1, correlated to "cell development" (κ=0.87)
Cellular stress	4.00	Response to stress	3.1E-6
		Response to wounding	3.6E-3
		Response to external stimulus	5.9E-2
		Defense response	2.0E-1
		Inflammatory response	4.5E-1
Cell cycle	3.8	Regulation of cell cycle	3.7E-3
Intracellular vesicle transport	3.76	Golgi vesicle transport	2.5E-4
		ER to Golgi vesicle-mediated transport	1.1E-2
Biotic stimulus	2.57	Response to biotic stimulus	3.8E-2
Tissue healing	2.08	Wound healing	9.1E-2
		Hemostasis	1.2E-1
		Blood coagulation	1.7E-1
		Regulation of body fluid levels	3.5E-1
		Platelet activation	4.9E-1
		Complement and coagulation cascades	8.8E-1

Table 4

Key cluster	EASE-score	Sub-cluster	P-value (Benjamini)
Transcription factor binding	3.25	Transcription factor binding	3.0E-2
		Trascription cofactor activity	6.1E-2
Apoptosis	2.29	Regulation of apoptosis	2.8E-1
		Cell development	6.8E-1
		Cell differentiation	9.9E-1
Cellular stress	0.56	Response to stress	1.0E-2
		Response to wounding	9.9E-1
		Response to external stimulus	5.2E-1
		Defense response	9.3E-1
		Inflammatory response	9.7E-1
Cell cycle	3.15	Regulation of cell cycle	2.9E-2
Intracellular vesicle transport	3.31	Golgi vesicle transport	6.3E-3
		ER to Golgi vesicle-mediated transport	3.0E-2
Biotic stimulus	1.5	Response to biotic stimulus	5.2E-1
Tissue healing	No representation	Wound healing	-
		Hemostasis	-
		Blood coagulation	-
		Regulation of body fluid levels	-
		Platelet activation	-
		Complement and coagulation cascades	-

Table 5

Hip fractures in patients day 7 vs. 1 HF(7vs1)				
Gene-ID	log FC	Adj. P. Val (BH)	Gene list	Gene function
210512_s_at	-2,01066	0,00038	VEGFA	Angiogenesis
202337_at	-0,70716	0,00049	PMF1	Osteogenesis
205207_at	-0,59814	0,017	IL6	Inflammation
209201_x_at	-1,14702	0,027	CXCR4	Stem cell attraction
203085_s_at	0,642476	0,042	TGFB1	Osteogenesis
Hip fracture in patients day 42 vs. 1 HF(42vs1)				
Gene-ID	log FC	adj. P. Val (BH)	Gene list	Gene function
210512_s_at	-2,10542	0,00022	VEGFA	Angiogenesis
202337_at	-0,59411	0,0028	PMF1	Osteogenesis
205207_at	-0,61017	0,014	IL6	Inflammation
209201_x_at	-1,28274	0,017	CXCR4	Stem cell acttraction

Figure 1:

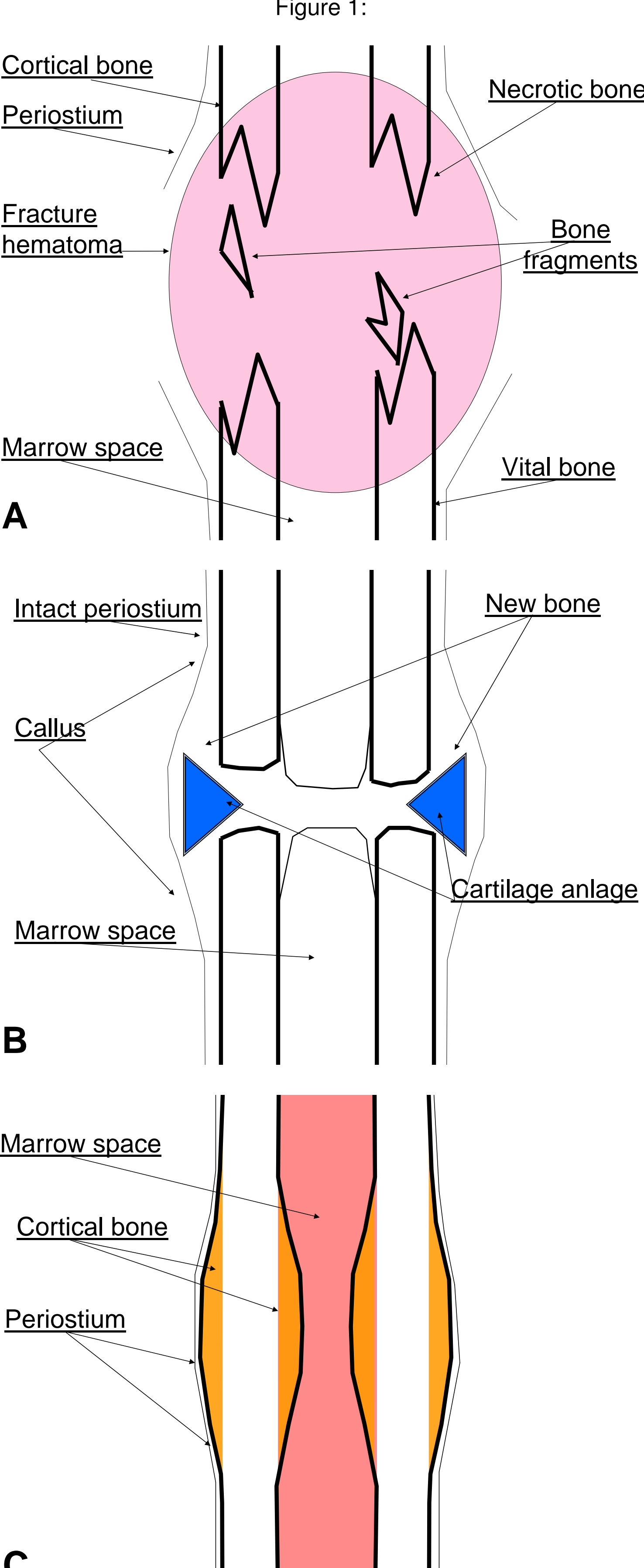


Figure 2:

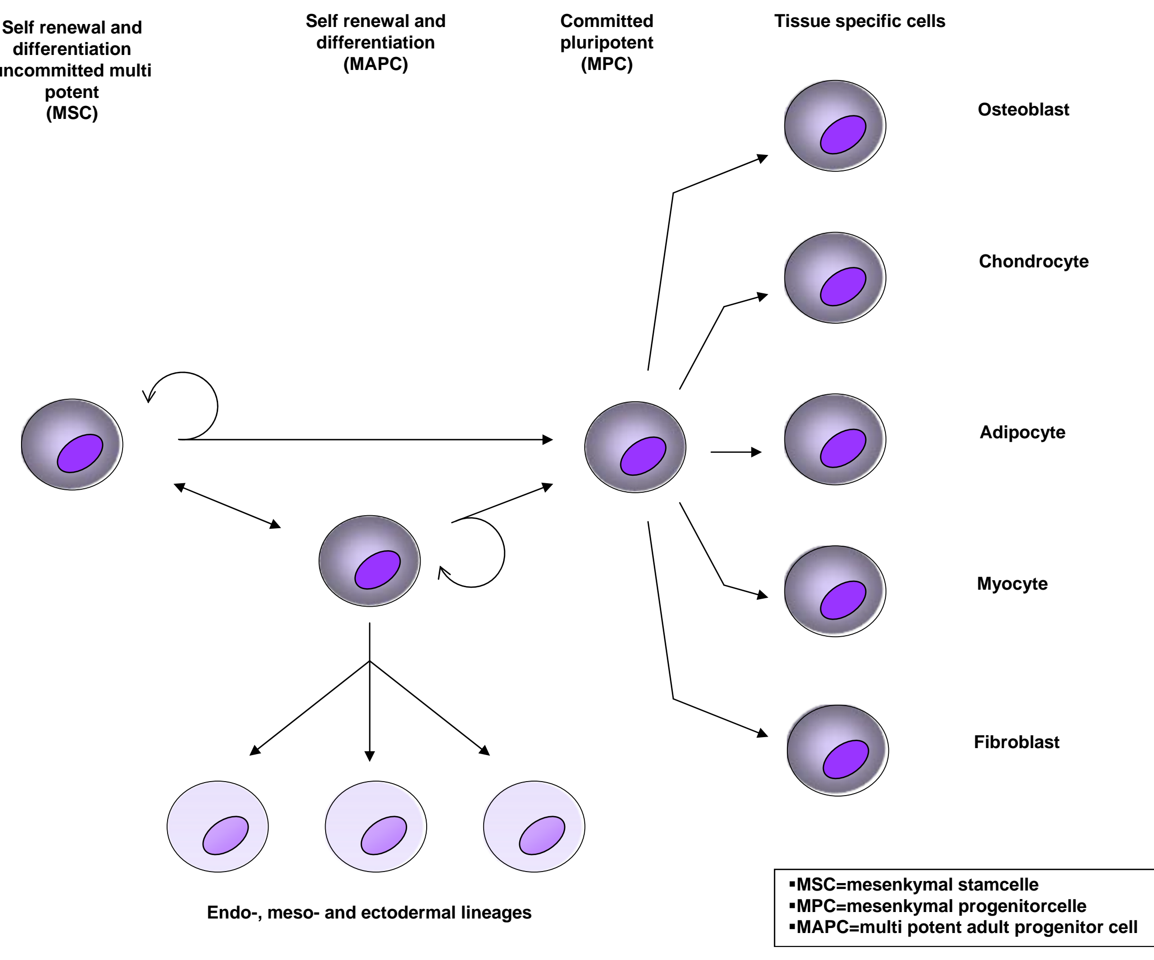
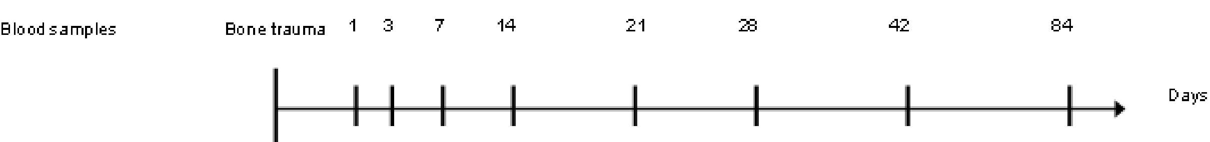
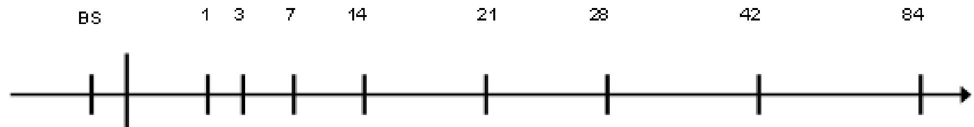


Figure 3:

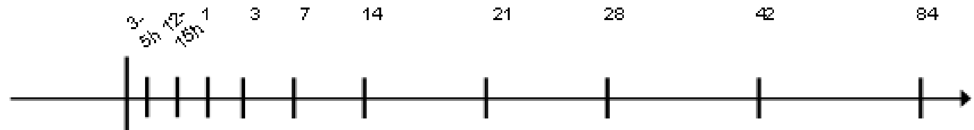
Cohort 1 AF, HF and THR



THR from cohort 2



HF from cohort 2



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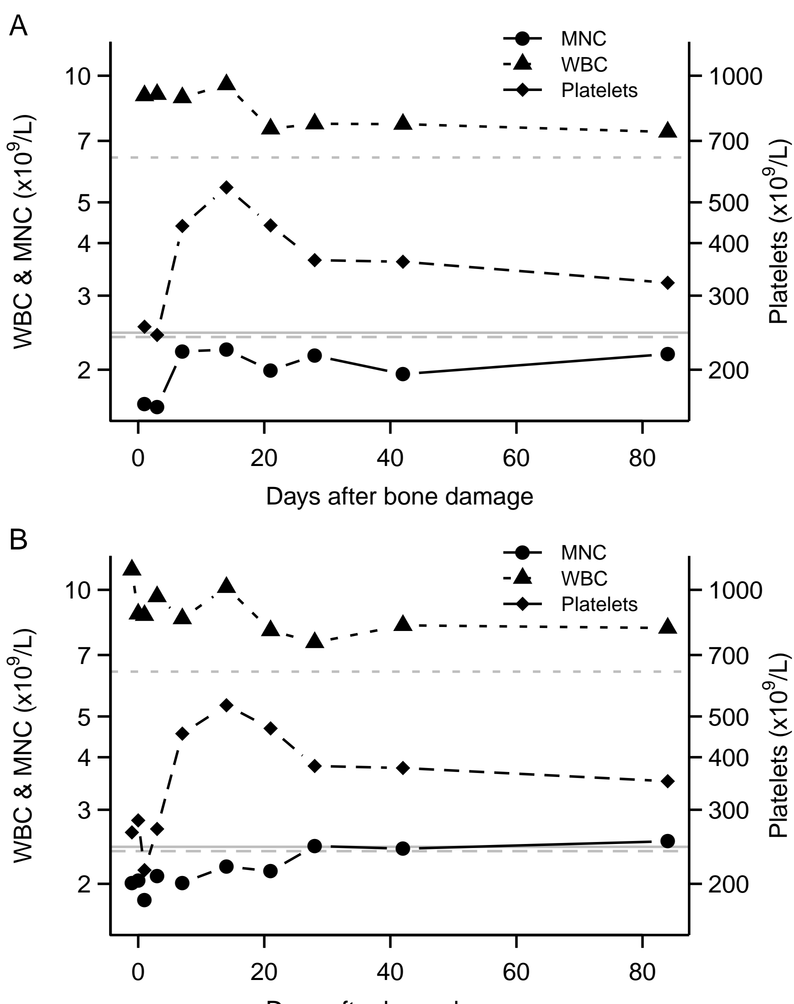


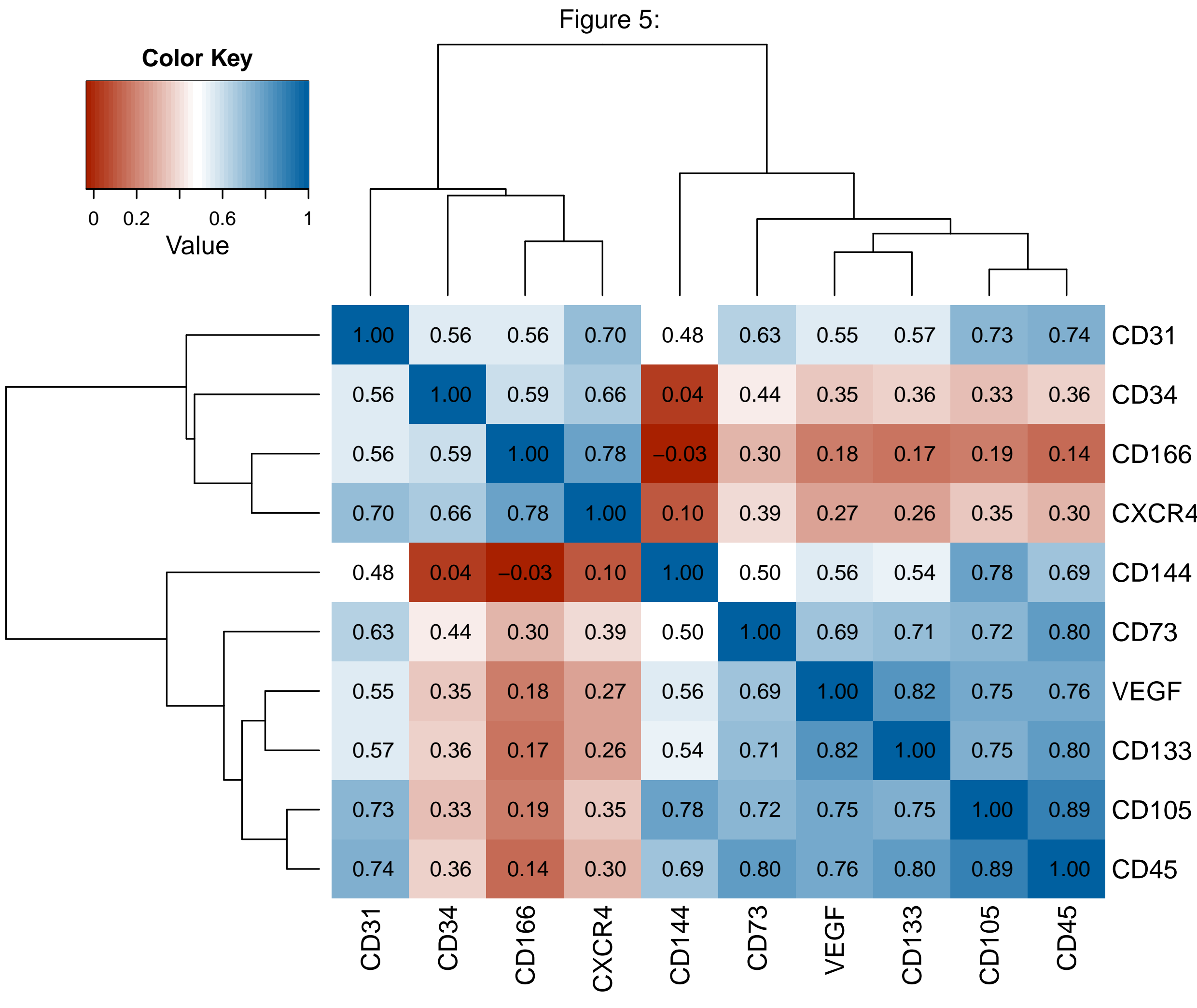
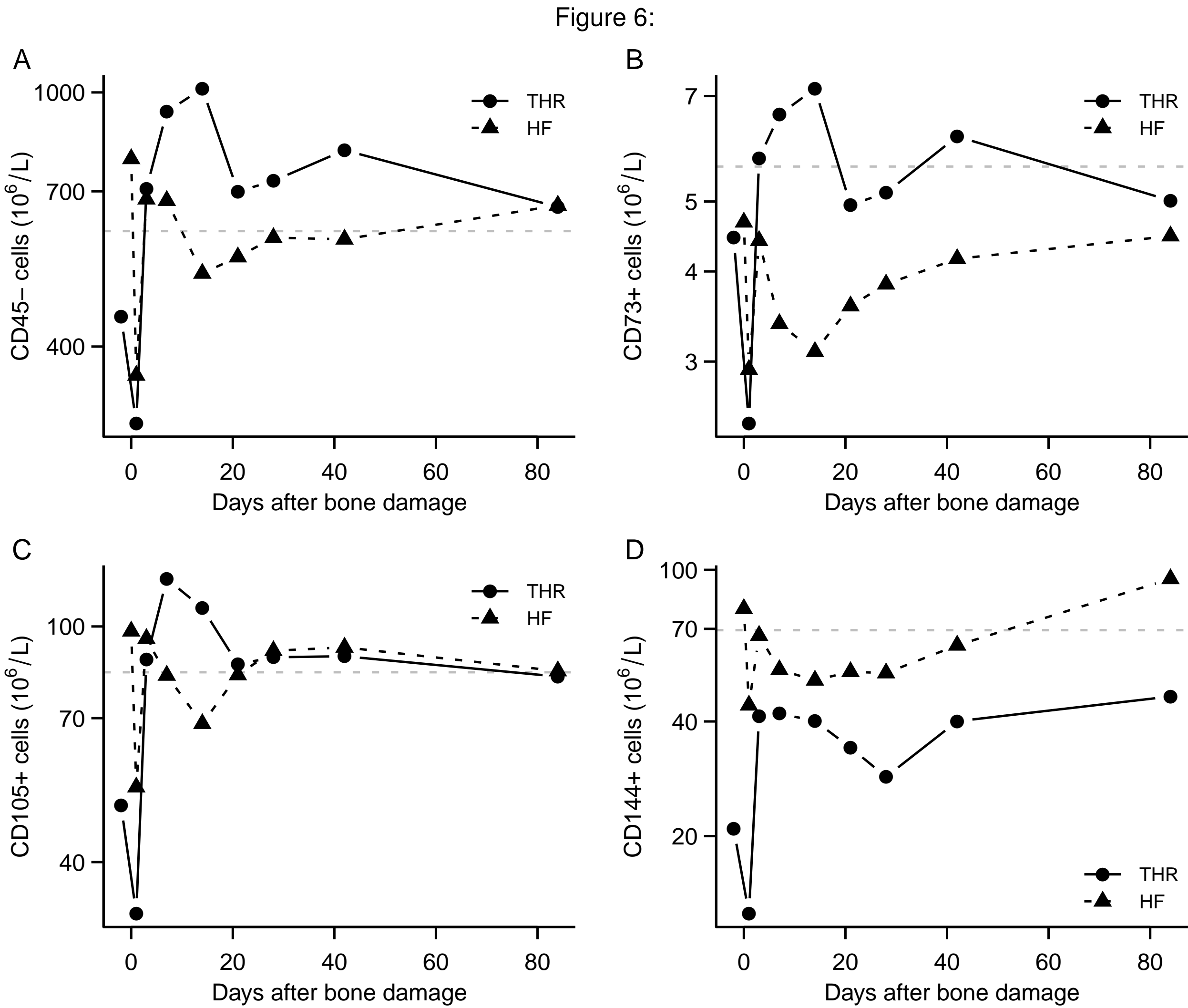


Figure 5:
Color Key
0 0.2 0.6 1
Value
CD31 1.00 0.56 0.56 0.70 0.48 0.63 0.55 0.57 0.73 0.74
CD34 0.56 1.00 0.59 0.66 0.04 0.44 0.35 0.36 0.33 0.36
CD166 0.56 0.59 1.00 0.78 −0.03 0.30 0.18 0.17 0.19 0.14
CXCR4 0.70 0.66 0.78 1.00 0.10 0.39 0.27 0.26 0.35 0.30
CD144 0.48 0.04 −0.03 0.10 1.00 0.50 0.56 0.54 0.78 0.69
CD73 0.63 0.44 0.30 0.39 0.50 1.00 0.69 0.71 0.72 0.80
VEGF 0.55 0.35 0.18 0.27 0.56 0.69 1.00 0.82 0.75 0.76
CD133 0.57 0.36 0.17 0.26 0.54 0.71 0.82 1.00 0.75 0.80
CD105 0.73 0.33 0.19 0.35 0.78 0.72 0.75 0.75 1.00 0.89
CD45 0.74 0.36 0.14 0.30 0.69 0.80 0.76 0.80 0.89 1.00
CD31 CD34 CD166 CXCR4 CD144 CD73 VEGF CD133 CD105 CD45

Figure 6:



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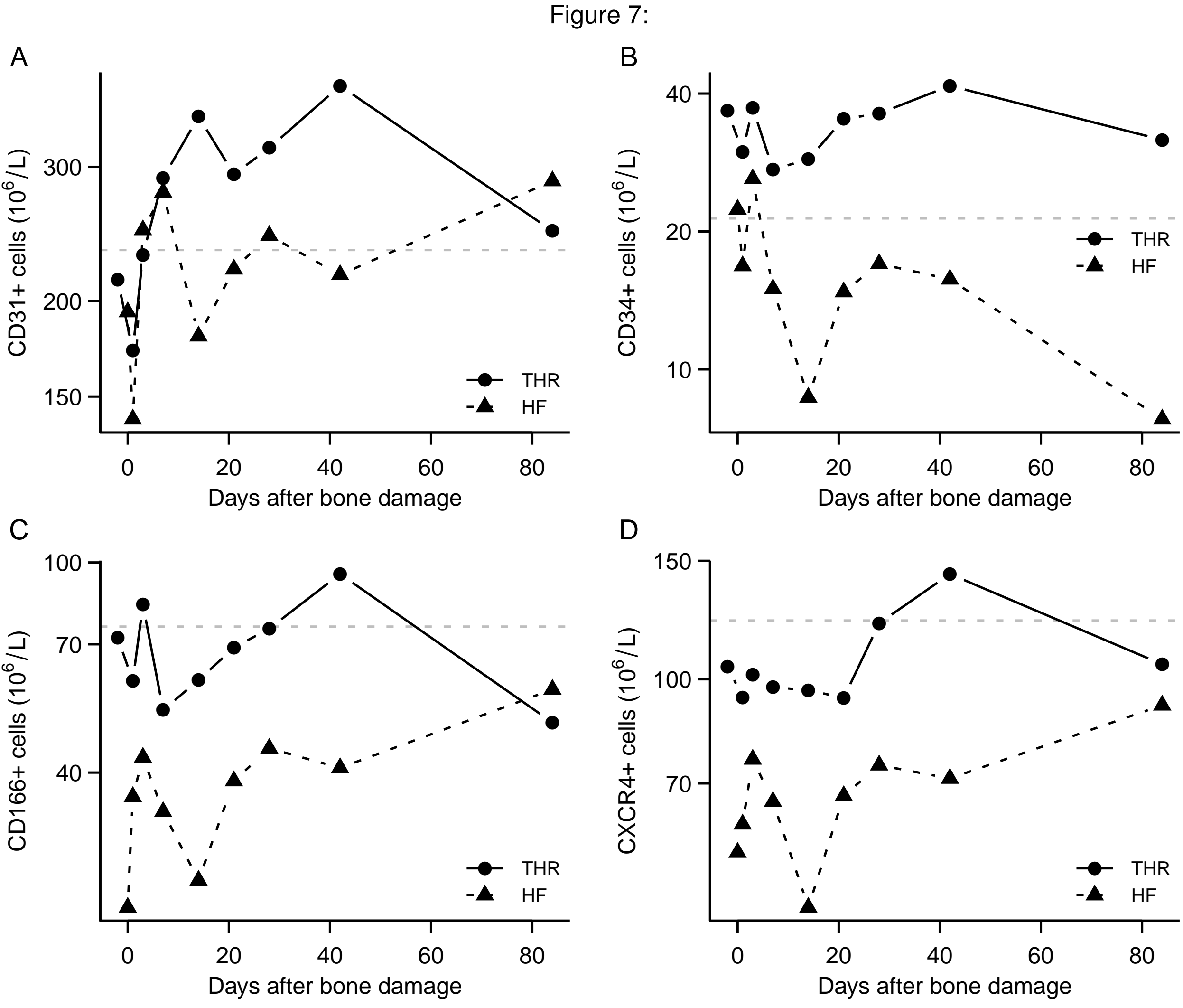

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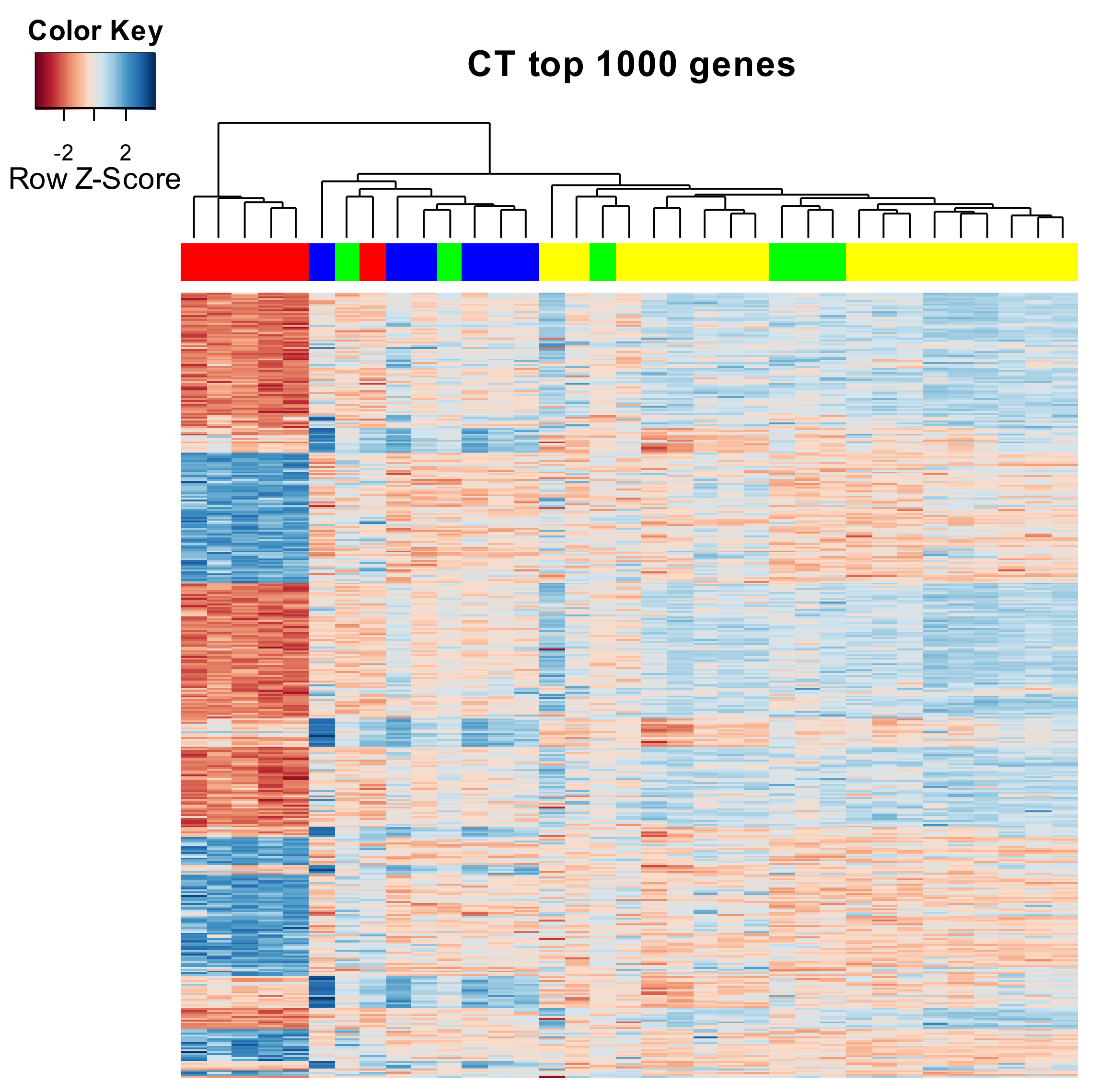

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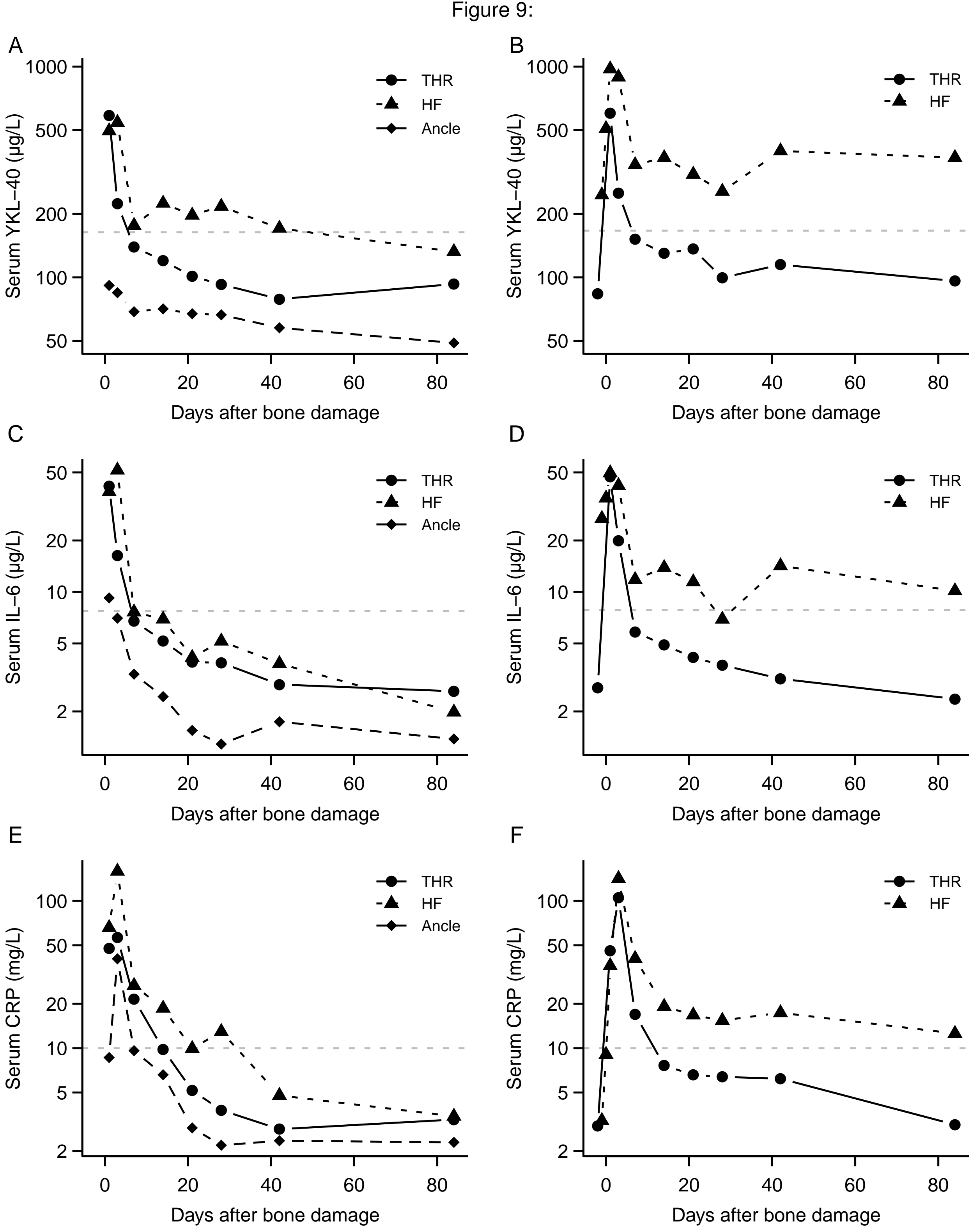

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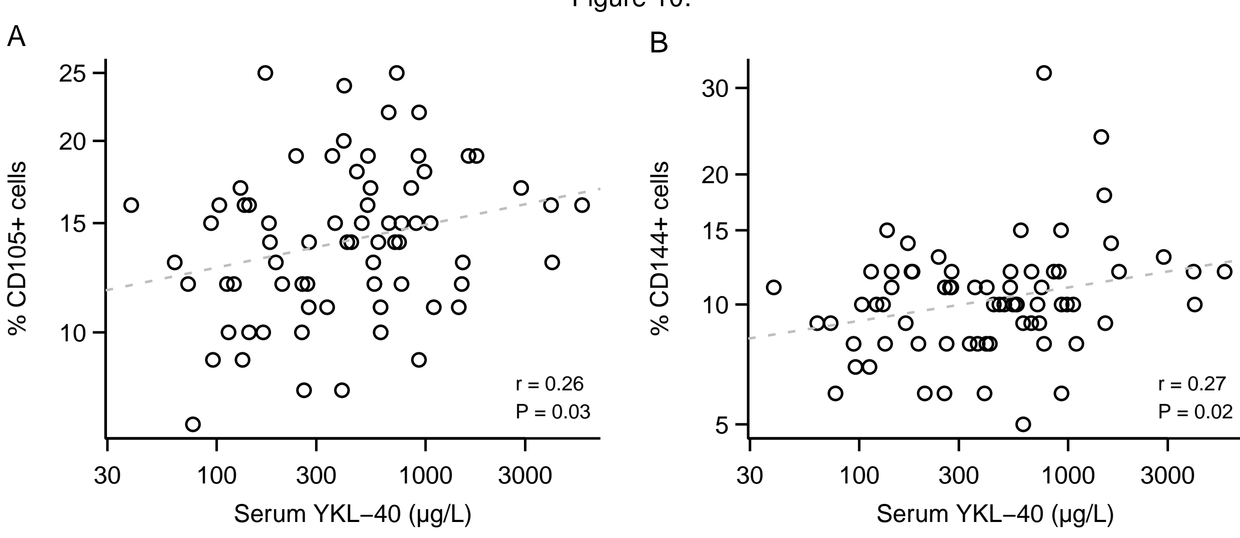

Dansk titel på ph.d.-afhandlingen:

ANALYSER AF CIRKULERENDE UMODNE MESENCHYMALE CELLEFORSTADIER OG UDVALGTE CYTOKINER UNDER KNOGLEREGENERATION

Resumé på dansk

Ved knogleskade frigives en række aktiveringsproteiner til blodbanen. Disse proteiner stimulerer en population af umodne celler i knoglemarven, såkaldte mesenkymale stam- eller progenitorceller, som frigives til blodbanen og specialiseres til modne knogle-, brusk-, fedt og bindevævsceller lokalt ved knoglebruddet.
Formålet med dette projekt er at karakterisere og kvantificere celler, proteiner og vækstfaktorer, som er nødvendige for knogleheling og som cirkulerer i blodbanen efter en knogleskade. Materialet omfatter blodprøver med cirkulerende mesenkymale progenitorceller og relaterede proteiner fra i alt 50 patienter med friske planlagte operative indsættelser af totale hofteproteser eller hoftenære lårbensbrud eller ankelbrud.
Cellernes fænotype karakteriseres med flow cytometri, som kan bestemme tilstedeværelsen af overflademolekyler, såkaldte overflademarkører. Denne analysemetode gør det muligt at identificere og kvantificere andelen af umodne mesenkymale progenitorceller i en heterogen population af cirkulerende mononukleære celler.
Vores analyser påviser at de umodne ikke-bloddannende celler i blodbanen omfatter to subpopulationer, som ændres forskelligt efter knogleskaden. Overflademarkørerne i den ene af subpopulationerne indikerer at disse kan være relaterede til modne og umodne endothelceller, mens cellerne i den anden subpopulation kan være relaterede til umodne mesenkymale celler.
Cellernes engagement i den posttraumatiske inflammation og regenerative kar- og knoglenydannelse understreges ved en kvantitativ analyse af de gener, som udtrykkes anderledes efter knogleskaden. Denne genanalyse er udført med Microarray gen analyse teknik. Da det overordnede genudtryk ændres voldsomt efter et knogletraume, for med tiden at returnere mod det normale, vidner dette om en traume-relateret rolle for disse isolerede mononukleære celler.
Genanalyserne omfatter også en gruppering af generne i funktionelle grupper, som med en vis sandsynlighed angiver cellens overordnede funktion. Denne analyse påviste en sandsynlighed for at disse cellers overordnede funktion var relateret til den posttraumatiske regeneration igennem et ændret udtryks-niveau af gener med kendt relation til knogleheling, kar-nydannelse og inflammation, som alle er vigtige elementer af knogleregeneration.
Vi kvantificerede vækstfaktoren YKL-40 og de to inflammatoriske cytokiner IL-6 (Interleukin 6) og CRP (C-reaktivt protein) med ELISA teknik.
Disse analyser viste en klar posttraumatisk korrelation mellem størrelsen af knogletraumet og både de inflammatoriske markører og YKL-40. Yderligere var det postinflammatoriske YKL-40 niveau afhængigt af om knogleskaden var operativ eller traumatisk. YKL-40 niveauet korrelerede positivt til koncentrationen af de cirkulerende umodne mesenkymalt associerede celler, under den tidlige knogledannelse, men kun i patienter med brækkede lårben, ikke i patienter med planlagte operative udskiftninger af hofteleddet.
Vi mener at vores model er velegnet til at undersøge det posttraumatiske respons, da denne har påvist en posttraumatisk forskel mellem de cellulære og humorale aspekter ved heling efter planlagte operative og traumatiske hofteskader. Vi har med denne model yderligere gjort nogle nye fund mht. hvilke cellulære subpopulationer, som cirkulerer efter et knogletraume samt en posttraumatisk korrelation mellem vækstfaktoren YKL-40 og en population af cirkulerende umodne mesenkymale celleforstadier i patienter med hoftebrud.

A CLINICAL MODEL STUDYING CIRCULATING CELLULAR AND HUMORAL BIOMARKERS INVOLVED IN BONE REGENERATION FOLLOWING TRAUMATIC LESIONS

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Running title: Circulating biomarkers during bone regeneration

Number of words: Abstract: 356; Manuscript: 4349

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Number of tables: 5

Key words: Bone regeneration, fracture, gene expression, mesenchymal progenitor cells, biomarkers, YKL-40.

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(a) All persons designated as authors have qualified for authorship according to the ICMJE criteria (http://www.icmje.org/sponsor.htm). All authors listed below have approved the final submission for publication. The contributions of each partner who participated in this collaborative project are given below.

Hans Gottlieb designed the study and wrote the protocol, performed analysis, provision of biomaterial and patient data, data collection and interpretation and prepared the manuscript and revised it critically for important intellectual content.

Tobias W. Klausen assisted in data analysis and interpretation and revised the manuscript critically for important intellectual content

Martin Boegsted assisted in data analysis and interpretation and revised the manuscript critically for important intellectual content

Bo S. Olsen assisted in provision of biomaterial and patient data, data collection result interpretation and revised the manuscript critically for important intellectual content

Gunnar S. Lausten assisted in provision of biomaterial and patient data, data collection result interpretation and revised the manuscript critically for important intellectual content

Jens Kastrup assisted in study design, result interpretation and revised the manuscript critically for important intellectual content

Mette Nyegaard assisted in data analysis, result interpretation and revised the manuscript critically for important intellectual content

Karen Dybkaer assisted in result interpretation and revised the manuscript critically for important intellectual content

Julia S. Johansen performed analysis and interpretation and revised the manuscript critically for important intellectual content

Hans E. Johnsen is corresponding author/guarantor and coordinator, assisted in study design and data collection, analysis and interpretation, manuscript preparation and revised the manuscript critically for important intellectual content

(b) Disclosures of potential conflict of interest; No:□ Yes:□, please describe below.

Disclosure details: ____

Signature: ______________________ City and Date: ________________

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ABSTRACT

Background

Fracture healing includes formation of cartilage, blood vessels and bone, which involves circulating progenitor cells, cytokines and growth factors in a complex homeostasis of tissue regeneration. Here we describe a clinical fracture model for the study of circulating cellular and humoral variables by a time dependent multiparametric approach.

Materials and Methods

Two cohorts with a total of 50 patients, with either ankle- or hip fracture or planned hip replacements were included as was a group of 17 healthy controls. Blood samples were timely collected during the post traumatic period and analysed for non haematopoietic mesenchymal cells by multiparametric flow cytometry (MFC), for global gene expression profiling (GEP) by micro array and serum biomarkers by immunoassays (ELISA). Integrative analysis was performed to identify patterns of biological variables with potential impact on cartilage, blood vessels and bone regeneration.

Results

Posttraumatic levels of circulating white blood cells and platelets as well as YKL-40, IL-6 and CRP varied biphasic and correlated to the magnitude of bone traumas.

Analytic MFC identified two $CD45^{neg}$ minor circulating compartments which in a time dependent manner simultaneously expressed varying degrees of CD105, CD133, CD 73, VEGF-R, CD144, or CD31, CD34, CD166, CXCR4, respectively - supporting the hypothesis that different mesenchymal subsets may be involved in fracture healing.

Blood mononuclear cell samples *analysed by GEP* identified large posttraumatic changes in gene expression in circulating MNCs from patients with hip fractures, functional clustering of these genes in relation to inflammation, cellular activity and cellular stress and significant expression of specific genes with known relation to inflammation, bone regeneration and angiogenesis.

Serum levels for YKL-40 analysed by *ELISA* identified variations in parallel with cellular changes with levels depending on the size of lesion i.e. higher in hip traumas compared to patients with ankle fractures (MNC: p=0.0006; YKL-40: p=0.0004). Serum YKL-40 differed significantly between traumatic- and planned bone traumas during bone formation (p=0.005).

Conclusions

We here describe the time dependent posttraumatic cellular and humoral response and identified circulating potential mesenchymal progenitor compartments, reactive gene expressed in circulating mononuclear cells and involved inflammatory biomarkers YKL-40, IL-6, and CRP during bone regeneration according to type and magnitude of bone trauma.

INTRODUCTION

Bone fracture results in cell death, local ischemia, formation of a fracture haematoma and secretion of pro-inflammatory cytokines as IL-1, IL-6, IL-8 and TNFα from traumatized periosteum, macrophages and inflammatory cells locally and in the circulation. This inflammatory stage initiates a cytokine mediated repair cascade with a chemotactic effect on inflammatory cells, enhancing extra cellular matrix synthesis, stimulating angiogenesis and recruiting endogenous fibrinogenic cells to the fracture site. A subsequent stage involves angiogenesis stimulated by VEGF, PDGF and angiopoietin, meanwhile a soft cartilage containing callus replaces the fracture haematoma. From about day 7 committed osteoprogenitor cells in the periosteum undergo intramembranous ossification, which is the formation of new woven bone without a preceding cartilage anlage. During this regenerative stage the fracture site is divided into a central cartilage containing zone and a peripheral bone containing zone. The general belief is that the distance from the vascularized fracture ends and periosteum decides whether the mesenchymal progenitor cells differentiate into bone or cartilage. The following stages of endochondral ossification in the central zone of the fracture haematoma involves hardening of the callus by cartilage calcification, in growth of new blood vessels, cartilage removal by apoptosis and finally bone formation. Cytokines as IL-1, IL-6, RANKL, OPG and TNFα also regulate the later endochondral bone remodelling (1-5).

At the fracture site mesenchymal progenitor cells are attracted and stimulated by the micro environment withholding growth factors such as TGFβ, PDGF, BMP, IGF-1 and -2 to differentiate into bone or cartilage cells (5-8). Mesenchymal progenitor cells are a compartment of inactive, non-haematopoietic mononuclear cells with the potential ability to differentiate into several types of mature cells as chondrocytes, osteoblasts and endothelial cells. Such mesenchymal progenitor cells have been isolated from many tissues e.g. bone marrow, fat, muscle, skin and peripheral blood (8-12) . Mesenchymal progenitors are released from the bone marrow to the fracture site by the circulation, and the formation of new blood vessels infiltrating the calcified callus is a limiting step for bone regeneration (1;2;13;14).

Fracture regeneration has several similarities with prenatal bone development, which has lead to the idea that differentiation of mesenchymal progenitor cells is a congenital ability, (re-)activated by certain evolutionary basic humeral signals after tissue traumas (12;15-20). The central role of IL-6 in bone regeneration is underlined by high concentrations of this cytokine during inflammation (2-5;15;21-23), but IL-6 has also been documented to influence and quantify the later ossification (21;24-26).

C-reactive protein (CRP) is an acute phase protein produced by the liver under transcriptional control of IL-6 (27). CRP has been reported to peak the second day after fracture and normalize by the third week (23;28). CRP quantifies the type of bone trauma, treatment and postoperatively complications as deep wound infection or bronchopneumonia (29-32).

YKL-40 (also named chitinase-3-like-1 and human cartilage glycoprotein-39) is a heparin-, chitin- and collagen-binding lectin and a member of “mammalian chitinase-like proteins”. YKL-40 is produced by human embryonic stem cells (unpublished), embryonic- and fetal cells, (18), arthritic chondrocytes, inflammatory cells and endothelial cells. The exact biological functions of YKL-40 are unknown, but it is a growth factor for chondrocytes and fibroblasts, it modulates the rate of type I collagen fibril formation, and has been suggested to play a role in cell proliferation and differentiation, angiogenesis, inflammation and in remodelling of the extra cellular matrix. The YKL-40 expression rises after cartilage trauma in arthritic joints and is related to the initial repair response (33-39). Serum YKL-40 increases rapidly after a malleolar or tibia fracture followed by a decrease shortly after osteosynthesis (40).

The observation that solid tissues are colonized by organ-specific circulating blood cells suggests that specific tissue regeneration and repair may be feasible if we can regulate progenitor cells from the circulating blood into areas of injured or diseased tissue and to modulate maturation once these cells have reached the target tissue. The basic humoral mechanisms that lead to formation of bone are known but the cellular components are still not completely understood. Therefore, translational research, including clinical studies needs to be performed to develop cellular treatment strategies.

In this descriptive report we have focused on a clinical *in vivo* model for bone regeneration following traumatic bone fracture or lesions studied by a multiparametric approach integrating data from cytomics, genomics and proteomics. The objective of the study was to identify temporal changes of important circulating subsets involved in bone regeneration, guided by analysis of known humeral biomarkers.

PATIENTS, MATERIALS AND METHODS

Patient inclusions and exclusions

The experimental protocol was approved by the regional scientific ethical committee in Copenhagen (Number KA 05081). All patients received oral and written information and participated under signed informed consent.

A total of sixty-two patients were primary included in two prospective cohorts admitted to the Orthopaedic Department from July 2005 to May 2007 with an ankle- or hip fracture or a planned hip replacement due to osteoarthrosis. Patients with multiple fractures, severe cardiopulmonary disease, and operation within 6 months or known malignancy were excluded. Demographical data were registered from medical records included age, sex, type of bone damage, complications and co-morbidity (Table 1).

Of 37 patients included in cohort 1, five patients were later excluded (1 patient with ankle fracture refused to participate, 2 patients with hip factures died, 1 had dementia and 1 had two fractures at a later examination). This resulted in 13 patients with ankle fractures, 10 patients with hip fractures and 9 patients with planned hip replacement (Table 1). Of 25 patients included in cohort 2, seven patients were later excluded (2 patients had diagnosed cancer, 1 refused to participate because of religious belief, and 4 patients died). This resulted in 10 patients with planned hip replacements and 8 patients with hip fracture. In summary a total of 50 patients completed the study periods and include the study cohorts. We further included 17 healthy controls with the same age as the fracture patients (Table 1). The healthy controls did not have surgery or bone fractures within the last 6 months, severe cardiovascular morbidity or known malignancy.

Design, material and methods

Cohort 1 (N=32) had blood sampled day 1, 3, 7, 14, 21, 28, 42 and 84 days after bone trauma. Cohort 2 (N=18) samples were collected the day before operation and 3-5 hours and 12-15 hours after bone lesion with additionally blood samples taken after trauma as for cohort 1. Blood samples were drawn into 5 x 10ml ethylenediaminetetraacetic acid (EDTA) stabilized tubes. Serum was isolated from 10 ml venous blood in one 10 ml Serum Sep Clot Activator glass.

Between ½-3 hours after vein puncture stabilized blood samples were processed into mononuclear cells (MNC) from the pooled EDTA-blood in T75 flasks (Nunc™) that was

centrifuged in portions of 15 ml diluted blood 1:2 in PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH2PO4·H2O, 6.5 mM NA2HPO4·2H2O, pH 7.3 – 7.4; Hospital pharmacy, Copenhagen, Denmark) on 10 ml Lymphoprep in 50 ml Leucosep® filter glasses (Greiner bio-one, Germany) at 2100 rpm, 10 minutes. The interface containing primarily mononuclear cells (MNC) were harvested and washed with PBS twice. Isolated MNCs were counted and subsequently frozen in 95 % fetal bovine serum (FBS) and 5 % dimethyl sulfoxide (DMSO) in 1.8 ml tubes containing 1 ml with 10^7 cells per ml at -80°C as described previously (41;42).

Enumeration of blood cells and subsets

Full blood leucocytes (WBC) and platelets were enumerated on a "Bechman Coulter AC-T diff 2" before preparation of MNC. The number of MNC in blood were calculated from the concentration of WBC and fraction of granulocytes (G) by MNC = WBC 10^9/L x (1-G).

MNC subsets were identified and enumerated by multi parametric flow cytometry (MFC) analysis. A number of $1x10^7$ cells were washed in FACS-PBS+10% FBS with EDTA. The cell pellet was incubated 30 minutes with the panel of highly selected CD specific antibodies given in Table 2 coupled by a range of cytochromes consisting of fluorescein isothiocyanate (FITC), r-phycoerythrin (PE), peridinin chlorophyll protein (PerCP) and allophycocyanin (APC). Antibody conjugated cells were then washed once with FACS-PBS + 10% NCS + EDTA to remove unbound antibody. A panel of monoclonal antibodies, including anti-CD45 to exclude haematopoietic cells and anti-CD31, -CD34, -CD73, -CD105, -CD133, -CD166, -CD144, -VEGFR-2 and -CXCR4 (CD184) to differentiate by appropriate analysis gates were used to identify and enumerate mesenchymal cell subsets.

The remaining cell pellet was mixed with 350µl FACS-PBS + 10%NCS + EDTA and applied for analysis by a four colour flow cytometer (FACS Calibur, BD bioscience). Antibody marked MNC were gated in a forward-/side scatter dot plot (Region 1). The strict $CD45^{neg}$ cells were defined (Region 2). Cells in region 1 and 2 were considered to be strict $CD45^{neg}$ non-haematopoietic cells with low granularity. The $CD45^{neg}$ cells were subsequently analyzed for presence of other membrane CD markers given in Table 2 and enumerated following subtraction of the corresponding negative controls. After acquisition of at least 50 000 cells per PB sample, analyses were considered as informative when adequate numbers of events (>100, typically 3-400) were collected in the enumeration gates.

Absolute cell numbers were calculated by reference leucocyte counts and healthy controls persons had a circulating level of non-haematopoietic cells identified as $CD45^{neg}$ of 0.66 x 10^9/L (0.3 x 10^9/L–1.2 x 10^9/L).

The concentration of subsets was performed by a two step calculation: first, the fraction of CD45^{neg} MNC (%) x blood concentration of MNC (10^9/L) = blood level of CD45^{neg} 10^9/L and second, this level of CD45^{neg} 10^9/L x CD % = blood level of a non haematopoietic CD subset.

Gene expression analysis by Micro array technique

Global gene expression profiling was performed on thawed circulating MNCs taken i) from 6 patients before operation, day 7 and 42 after planned hip replacements and ii) from 6 patients 3-5 hours, day 7 and 42 after hip fractures.

Micro array analysis was performed on isolated MNC lysed with Trizol (Invitrogen Trizol ® Reagent Cat. No. 15596-026) and total RNA was purified on columns (MirVana™ miRNA isolation Cat. No. 1561 Ambion). With a Poly-A-tail accepting primer, a cDNA strand was synthesised. A T7 RNA polymerase was used to generate the biotin labelled cRNA via an in vitro transcription (GeneChip®Expression 3´-Amplification Reagents for IVT Labelling, Affymetrix). 3 µg isolated total RNA was amplified and resulted in 25 µl biotin labelled cRNA, from which 20 µg were put in a hybridization cocktail and placed on HGU 133 +2.0 Affymetrix gene chips. The gene chips were read on a scanner and expression data stored as .cel-files in a data base.

The subsequent analysis was performed using Bioconductor packages (43) which are add-on modules for the statistical package R, (R Development Core Team, 2004).

As we experienced that the two treatment groups have considerably variations in their gene-expression patterns over time, we choose to normalise and analyse the three data-sets separately. Only in this way we are sure to maintain interesting time variations in each group during normalization. A batch effect across the treatment groups was noticed. We adjusted for this effect by centralising each group towards the grand mean of all probes.

The quality of the raw data was inspected by RNA-degradation plots and histograms for each patient by routines from the Bioconductor package arrayQualityMetrics (44). No slides showed poor quality (data not shown).

Background correction and normalisation was carried out by the Robust Multichip Average procedure (RMA) found in the Bioconductor package "affy" (45). We made an unspecific prefiltering for the hip fracture and hip prosthesis groups with nsFilter from the Bioconductor package gene filter. This procedure considerably brought down the number of probes. No filtering of the healthy control group was performed as this group acts as control in later comparisons.

For each treatment group a linear mixed effects model was fitted to the logarithm of the gene expressions, with patients modelled as a random effect to take into account the inter-person correlation. Time was modelled as a fixed effect. The Bioconductor package Limma (46) was used to fit the model. Differentially expressed genes between time points were identified by consideration of moderated t-tests for relevant contrasts. The moderated t-test is based on empirical Bayes analysis, and is equivalent to shrinkage of estimated sample variances towards a pooled estimate, resulting in more stable inference when the number of micro array experiments is small. Due to the high number of false positives introduced because of multiple testing, we used the Benjamini-Hochberg (BH) q-values to control or estimate the false discovery rate. The meaning of "BH" q-values is as follows. If all genes with q-value below a threshold, say 0.005, are selected as differentially expressed, then the expected proportion of false discoveries in the selected group is controlled to be less than the threshold value, in this case 0.5%.

Comparisons between the hip fractures and hip prosthesis groups versus the healthy control group were performed by heatmaps. We used the heamap.2 function from the R-package gplots. The hierarchical cluster analysis indicated in these heatmaps was based on the R-function hclust using average as the agglomeration method and Euclidian distance as dissimilarity measure.

Biomarker YKL-40, IL-6 and CRP analysis

Samples were analyzed without clinical knowledge of the patients. Serum concentrations of YKL-40 and IL-6 were determined by Enzyme-Linked ImmunoSorbent Assay (ELISA) (YKL-40: Quidel, CA, USA; IL-6: Quantikine HS, high sensitivity, R&D Systems, Oxon, UK) in accordance with the manufacturer's instructions. The detection limit for YKL-40 was 20 μg/l, and the intra- and inter-assay coefficient of variations (CVs) were <5% and <6%, respectively. The detection limit for IL-6 was 0.17 ng/l, and the intra- and inter-assay CVs were <6.6% and <8.9%, respectively. All samples and standards were analysed in duplicates, and samples from each patient were analyzed on the same ELISA plate. Three (IL-6) and two (YKL-40) internal control samples were analysed on each plate to confirm assay precision.

The reference interval for serum YKL-40 and IL-6 were determined in healthy subjects with no signs of pre-existing disorders such as joint, liver, metabolic or endocrine disease or malignancy and no medication. The median serum YKL-40 in 245 healthy adults (134 women and 111 men, median age 49, range 18 to 79 years) was 43 μg/l (range 20 - 184 μg/l; 5th - 95th percentile: 20 - 124 μg/l). The median serum IL-6 in 318 healthy adults (122 women and

196 men, median age 47, range 18 to 64 years) was 1.4 ng/l (range 0.25 - 22.5 ng/l; 5th - 95th percentile: 0.51 - 4.92 ng/l).

Serum concentrations of C-reactive protein (CRP) was analysed on a KONELAB 60 apparatus that measures immune complexes with turbidimetry at 340 nm in accordance with the manufacturer's instructions. The antibody supplier is DakoCytomation, Glostrup, Denmark. The lower measuring limits are 3 mg/L for normal CRP and 0.2 mg/L for high sensitive C-reactive protein (hs-CRP). Intra-assay CV was 6.3%. Serum CRP values from 0-90 mg/L were measured using the hs-CRP method. Serum CRP values from >90 mg/L were measured using the normal CRP method. CRP values <10 mg/l is considered normal.

Statistical analysis

The statistical calculations were done by SPSS (SPSS statistical software system, version 17.0, Chicago, IL, USA) and R (R project for statistical computing, version 2.8.0, Vienna, Austria). All tests were two-sided and p-values less than 0.05 were considered significant. Skewed variables were log transformed to obtain normality when needed.

Comparison between groups was done by t-test or Mann-Whitney's test when the normality assumption was violated and comparison within groups were done by paired t-test or Wilcoxon's signed rank test when the normality assumption was violated.

Variations over several time points were calculated by a mixed-effect model, using the R package nlme. The time/visit variable was treated as a factor / ordinal variable.

Correlations were calculated using Spearman's rho test. The mean values of serum YKL-40, -IL6 and -CRP during the 12 weeks study were calculated as the area under the curve (AUC) values and using 8 time points for patients in cohort 1 (i.e. at baseline and after 1, 3, 7, 14, 21, 28, 42, and 84 days), 9 time points for patients with planned total hip replacements- and 10 time points for patients with hip fractures in cohort 2. Since YKL-40 increases with age an age-related reference interval was calculated on the logarithmically (log) transformed serum YKL-40 values of the healthy controls (36). The 95% percentile was chosen as the cut-off point.

RESULTS

Blood counts following fracture and during regeneration

The number of circulating leucocytes and platelets changed during bone regeneration as did non-haematopoietic cells defined as CD45^{neg} cells with an initial reduction followed by a rise day 1-2 and an overshoot in the following week (Figure 1).

Enumeration of non-haematopoietic subpopulations in circulation

Multiparametric flow cytometry analysis of circulating cells in patients with surgical or traumatic bone traumas revealed the phenotype of two homogeneous clusters of non-haematopoietic cells without the common leukocyte marker CD45 (Figure 2).

The CD45^{neg}/CD73/CD105/CD144^{pos} (cluster 1) declined sharply the first day after bone trauma in patients with planned hip replacements or hip fractures, indicating a posttraumatic extra vascularisation from the circulating blood into reactive areas. The compartment in cluster 1 peaked earlier in patients with hip fractures than in patients with planned hip replacements, followed by a plateau from day 21 to 84 in both groups (Figure 3A-D), possibly due to different regenerative responses in the two patient groups.

The level of CD45^{neg}/CD31/CD34/CD166/CXCR4^{pos} (cluster 2) cells were higher in patients with planned hip replacements than in patients with hip fractures throughout the study period ($p=0.049$, $p=0.002$, $p=0.02$ and $p=0.047$) (Figure 4A-D).

Thus, the first hours following surgery or fracture the level of CD45^{neg} cells is reduced to a value close to zero (Figure 3) in parallel with the biomarkers analysed (vide infra) and subsequently increases to normal levels during regeneration.

Micro array gene expression in circulating MNC

With a false discovery rate (FDR) at 0.5%, 1462 genes changed expressions level from day 1 to 7 in patients with hip fractures, and 1456 when comparing day 1 with day 42 after fracture. A heatmap (Figure 5) including the 1000 most varying genes for the hip fracture over the time-period and the healthy controls was used to illustrate that the gene expression in the patients changes violently within the first 24 hours after the fracture and then normalizes towards the pre-fracture homeostatic equivalence during 42 days of bone regeneration. Even though the numbers of genes that changed expression level from day 1 to respectively day 7 and 42 were almost similar, the functional clustering of the up- or down regulated genes differed (Table 3 and 4). Gene clusters with influence on inflammation, cellular activity and

cellular stress played a more important role for the total gene function of the circulating MNCs at day 7 than at day 42, documented by higher EASE-scores of the clusters correlated to bone regeneration at day 7 than at day 42 and lower p-values of the sub-clusters. The key-cluster "Tissue healing" was not significantly present at day 42, as it was on day 7 (Table 3 and 4). The functional cluster analysis documented a stronger involvement in inflammation, cellular activity and cellular stress due to tissue stress on day 7 than on day 42.

Serum biomarkers following fracture and during regeneration

Patients with hip fractures or planned hip replacement had an initially higher serum YKL-40 concentration than patients with ankle fractures (median 710 μg/l, range 102-1478 μg/l vs. 77 μg/l, 38-345 μg/l, P=0.0004) from day 1-7. The posttraumatic serum YKL-40 increased in patients with hip fractures (P=0.0001) and patients with planned hip replacements (P<0.0001) (Figure 6A and 6B).

Serum IL-6 rose from trauma to day 1 (P<0.0001) and declined in a similar pattern in all three patient groups, indicating that IL-6 quantifies the same posttraumatic inflammatory process (Figure 6C and 6D). IL-6 in patients with hip fractures and patients with planned hip replacements were significantly higher than in patients with ankle fractures at day 1 (P<0.0001), indicating a relation to the magnitude of the traumatized bone (Figure 6C). As for YKL-40, IL-6 values peaked at day 1 in patients with planned hip replacements and patients with ankle fractures, but at day 3 for patients with hip fractures (Figure 6C). There were no differences in IL-6 values between patients with hip fractures and patients with planned hip replacements from day 14 to 42 (p=0.4), which is different from YKL-40.

Changes in s-CRP are known to quantify ongoing inflammation as IL-6. Serum CRP rose (p<0.0001), but with a significant difference between patients with hip fractures or patients with planned hip replacement compared to patients with ankle fractures (p=0.0021) (Figure 6E). Changes in s-CRP is considerable in patients with hip fractures (P<0.0001) and patients with planned hip replacements (P<0.0001) (Figure 6E and 6F). The changes in CRP were not higher in patients with hip fractures than in patients with planned hip replacements (p=0.13 and p=0.08).

DISCUSSION

Knowledge of bone repair mechanism is mainly based on animal studies as an essential tool to analyze the biology of fracture healing. Accordingly, numerous mammalian species, ranging from the mouse to the horse, have been introduced as models. Nonetheless, differences in the anatomy and metabolism of animals compared to humans must be considered a limitation in transfer of the experimental data to the clinical situation. Therefore, this study addresses a human model of fracture repair with special emphasis on the time dependent involvement of circulating cellular and humoral biomarkers (4;5;7;22).

The objective was to measure the initial and later changes of the number of specific compartments of mononuclear cells circulating in peripheral blood and serum levels of IL-6, CRP and YKL-40 in patients during inflammation and following bone regeneration after surgical- or traumatic bone injuries. Patients with traumatic hip or ankle fractures regenerates through intra-membranous and endochondral ossification, in contrast to patients with total hip replacements due to osteoarthritis, who have their implants fixated through cementation, which involve very little if any formation of new bone. The peri-prosthetic bone resorption leading to aseptic loosening of the prosthesis maybe due to a local cellular immunological reaction (47-49).

Multiparametric flow cytometry analysis of circulating cells generated two homogeneous $CD45^{negative}$ clusters. The cluster integrity of the two MNC subpopulations was supported by similar changes over time for specific surface makers. One cluster consisted of $CD45^{neg}$ cells with presence of CD73, CD105 and CD144 known to be associated with mesenchymal progenitor cells (8;50;51). The second cluster consisted of $CD45^{neg}$ cells with presence of CD31, CD34, CD166 and CXCR4 (Figure 2). The surface markers CD31 and CD34 are associated with mature endothelial- and endothelial progenitor cells (52;53) involved in neo-angiogenesis of the ischemic traumatized fracture haematoma. Reperfusion of the ischemic fracture haematoma is important for conduction of blood born mesenchymal progenitor cells and the following endochondral ossification. CD166 is associated mesenchymal progenitor cells and cell adhesion (54;55). CXCR4 is a chemokine receptor present on cells that are drawn towards traumatized or ischemic tissue, which secretes SDF-1 (stromal cell derived factor 1) (19;56). The presence of these two surface markers on cells in the same cluster indicates a similar and related role in regeneration of traumatized- and ischemic bone.

Extended analysis of the posttraumatic cellular compartment by global micro array screening for *gene expression analysis* identifies a set of up- or down regulated genes involved in bone

regeneration, inflammatory and neo-angiogenesis. The magnitude of the posttraumatic changes in the gene lists from day 7 and 42, together with the pattern of gene expression going from highly abnormal towards normal homeostasis during the first 42 days after fracture also support the trauma-related role of the circulating MNCs (Figure 5). The functional role and involvement in actual bone regeneration, inflammation and neo-angiogenesis of these cells are further indicated by the functional cluster analysis of the up- or down regulation of certain benchmark genes within the gene lists at day 7 and 42 (Table 5) (57;58).

The *humoral inflammatory response* correlated significantly to the magnitude of traumatized bone, documented by larger changes in YKL-40, IL-6 and CRP in patients with hip fractures or planned hip replacements than in patients with ankle fractures after bone trauma. In contrast to IL-6 and CRP, the YKL-40 level was significantly higher in patients following hip fractures than patients with cement fixated planned hip replacements from day 14-42 in cohort 1 and 2. The high level of YKL-40 during bone formation in patients with hip fractures may indicate YKL-40 as a quantitative marker of endochondral ossification, independent of age. The central and interesting role of YKL-40 in bone traumas are underlined by the correlation between YKL-40 and the magnitude of the bone injury. The faster post traumatic decline towards normal YKL-40 values, in patients with planned hip replacements than in patients with hip fractures, could be due to the lesser bone regeneration after cement fixation than after traumatic bone fracture, caused by the chemical toxicity and heat generation of the bone cement (Figure 6A) (59-61).

IL-6 knockout-mice show delayed callus mineralization and bone remodelling (24). Treatment with combined IL-6 and PTH enhanced fracture healing in rats by enlarging callus volumes, completing unions and increasing the mechanical strength of regenerated fractures compared to controls (25). Finally IL-6 was elevated in rats during remodelling of regenerating bone (26). Prolonged elevation of IL-6 was reported to have an adverse effect on lower extremity function after hip fracture in rats, indicating that normal bone remodelling depends on normalization of IL-6 (21;26).

In conclusion, this report describes the posttraumatic cellular and humoral response and identified circulating potential mesenchymal progenitor compartments, reactive genes expressed in circulating mononuclear cells and involved inflammatory biomarkers YKL-40, IL-6, and CRP during bone regeneration according to type and magnitude of bone trauma. This model will be used to identify, sort and characterize the potential mesenchymal progenitor

cell compartment by in vitro culture studies and specific gene expression studies with the attempt to define the phenotype of involved stem and progenitor cells.

FIGURE LEGENDS

Figure 1A-B

Quantification of circulating leucocytes, mononuclear cells and platelets in cohort 1 as shown in A and cohort 2 as shown in B. Healthy controls had an average age of 71 years (63-81) and a mean number of circulating leucocytes of 6.53 x 10^9/L (4.1 x 10^9/L–8.8 x 10^9/L) and MNC of 2.53 x 10^9/L (1.19 x 10^9/L 3.57 x 10^9/L).

Figure 2

The $CD45^{neg}$ MNC clusters in two subpopulations according to the changing intensities of the specific surface markers during bone regeneration. The dendrogram illustrates the division of the surface markers in two clusters designated cluster 1 and cluster 2. The numbers in the squares are the correlation coefficients, which are also shown in the colour key.

Figure 3A-D

Changes in $CD45^{neg}$ MNC (A), $CD73^{pos}$, $CD45^{neg}$ MNC (B), $CD105^{pos}$, $CD45^{neg}$ MNC (C), $CD144^{pos}$ $CD45^{neg}$ MNC (D) (Cluster 1) during bone regeneration.

Figure 4A-D

Changes in $CD31^{pos}$, $CD45^{neg}$ MNC (A), $CD34^{pos}$, $CD45^{neg}$ MNC (B), $CD166^{pos}$, $CD45^{neg}$ MNC (C) and $CXCR4^{pos}$, $CD45^{neg}$ MNC (D) (Cluster 2) during bone regeneration.

Figure 5

The heatmap shows the standardised values of the 1000 most time-varying genes. Red are row-wise higher values and blue are row-wise lower values. The genes are row-wise sorted by a cluster algorithm according to their similarity with other genes. Likewise are the samples column-wise sorted by a cluster algorithm according to their similarity with other samples. Red are the samples at time 1, blue the samples at time day 3 and green the samples at time day 42. The yellow samples are the healthy controls.

Figure 6A-F

Change in YKL-40, IL-6 and CRP during bone regeneration. Figure A, C, E are YKL-40, IL-6 and CRP from cohort 1. Figure B, D, F are YKL-40, IL-6 and CRP from cohort 2. Dotted lines mark upper boundaries of normal levels.

The average levels of YKL-40 in patients with hip fractures from cohort 2 were 116 µg/L and 68 µg/L in patients with hip replacements. These patients had an average level of IL-6 at 3.35 µg/L. The average level of YKL-40 in patients from cohort 1 is 83µg/L. A CRP value <10 mg/L are considered as non-pathological.

TABLE LEGENDS

Table 1

Demographic and clinical data included age, sex, type of bone damage, complications and co-morbidity.

Table 2

The panel of highly selected CD specific antibodies used for identification and enumeration of subpopulation by multiparametric flow cytometry.

Table 3

Key- and sub-clusters of genes with possible relation to the ongoing posttraumatic inflammation or bone regeneration. The genes are differentially expressed at day 1 and day 7 in patients with hip fractures.

Table 4

Key- and sub-clusters of genes with possible relation to the ongoing posttraumatic inflammation or bone regeneration. The genes are differentially expressed at day 1 and day 42 in patients with hip fractures.

Table 5

Identification of Benchmark-genes expressed in blood MNC from in hip fractured patients.

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Table 1

Bone trauma	No. of pt. in./ex./ completed	Age/ years	Sex	Co-morbidity		Complications affecting bone regeneration	Total Blood samples
				total	diagnoses		
AF	14/1/13	47(22-68)	7M 6F	2/13	2 RD	3/13	8
HF (1)	14/4/10	77(60-89)	5M 5F	5/10	1 GI, ND, t.CP 1 RD,OP, t.ED 1 OP 1 OP, t.CP,ND 1 AS	6/10	8
THR (1)	9/9	70(44-84)	2M 7F	9/9 OA 3/9 nOA	1HT 1OP,RD,t.CP 1RD	1/9	8
HF (2)	13/5/8	85(75-90)	8F	7/8	1 DE 1 t.CP 1 OP 1 DE 1 OP,t.ED,RD 1 HP,RD 1 ND,DE	2/8	10
THR (2)	12/2/10	68(50-80)	2M 8F	10/10 OA 6/10 nOA	1 t.CP 1 t.CP 1 t.CP 1 HP,t.ED 1 HP, t.ED 1 HP	1/10	9

(1) Study 1. (2) Study 2. AF – ankle fracture, HF – hip fracture, THR – Total hip replacement. OA – osteo arthritis, t.CP – treated cardiovascular illness, HC – high cholesterol, t.ED – treated endocrine disease, DE – dementia, OP – osteo porosis, RD – rheumatoid disease, HP – high blood pressure, nOA – non osteo arthritic disease, GI – Gastro intestinal disease, ND – Neurological disease, AS – Artery sclerosis,

Table 2

Associated cell population	Cluster of differentiation	Antigen	Conjugation
Leukocytic common antigen	CD45	LCA	PerCP
Haematopoietic progenitor cell	CD34	Glycoprotein 105-120	APC
Haematopoietic progenitor cell	CD133	AC133	PE
Mesenchymal progenitor cell	CD73	Ecto-5'-nucleotidase	PE
Mesenchymal progenitor cell	CD105	Endolgin	FITC
Mesenchymal progenitor cell	CD166	ALCAM	PE
Endothelial cells	CD31	PECAM-1	FITC
Endothelial progenitor cell	CD144	VE-cadherin	FITC
	VEGFR-2	VEGF 2	PE
Injured and hypoxic tissue secreting SDF-1	CXCR4 (CD184)	CXCR4	PE
Negative control	IgG1(Mouse)		FITC, PE, PerCP, APC

Table 3

Key cluster	EASE-score	Sub-cluster	P-value (Benjamini)
Transcription factor binding	5.7	Transcription factor binding	4.4E-6
		Trascription cofactor activity	7.5E-4
Apoptosis	4.86	Regulation of apoptosis	1.7E-5
		Cell development	1.6E-2
		Cell differentiation	3.3E-1, correlated to "cell development" (κ=0.87)
Cellular stress	4.00	Response to stress	3.1E-6
		Response to wounding	3.6E-3
		Response to external stimulus	5.9E-2
		Defense response	2.0E-1
		Inflammatory response	4.5E-1
Cell cycle	3.8	Regulation of cell cycle	3.7E-3
Intracellular vesicle transport	3.76	Golgi vesicle transport	2.5E-4
		ER to Golgi vesicle-mediated transport	1.1E-2
Biotic stimulus	2.57	Response to biotic stimulus	3.8E-2
Tissue healing	2.08	Wound healing	9.1E-2
		Hemostasis	1.2E-1
		Blood coagulation	1.7E-1
		Regulation of body fluid levels	3.5E-1
		Platelet activation	4.9E-1
		Complement and coagulation cascades	8.8E-1

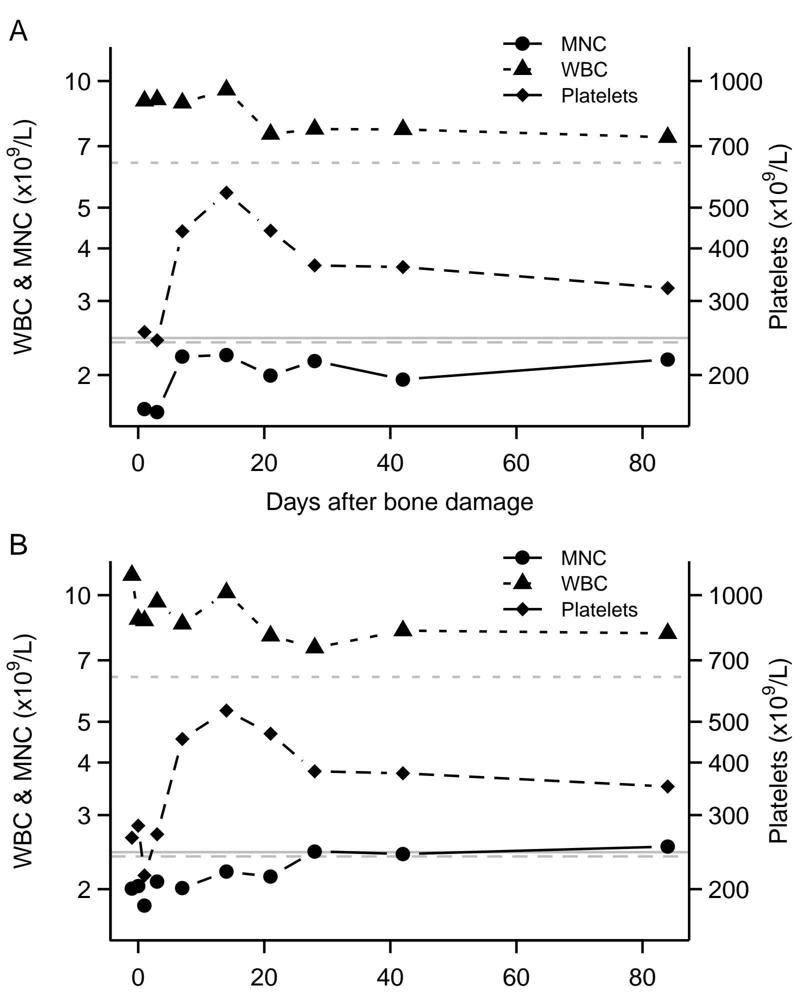
Table 4

Key cluster	EASE-score	Sub-cluster	P-value (Benjamini)
Transcription factor binding	3.25	Transcription factor binding	3.0E-2
		Trascription cofactor activity	6.1E-2
Apoptosis	2.29	Regulation of apoptosis	2.8E-1
		Cell development	6.8E-1
		Cell differentiation	9.9E-1
Cellular stress	0.56	Response to stress	1.0E-2
		Response to wounding	9.9E-1
		Response to external stimulus	5.2E-1
		Defense response	9.3E-1
		Inflammatory response	9.7E-1
Cell cycle	3.15	Regulation of cell cycle	2.9E-2
Intracellular vesicle transport	3.31	Golgi vesicle transport	6.3E-3
		ER to Golgi vesicle-mediated transport	3.0E-2
Biotic stimulus	1.5	Response to biotic stimulus	5.2E-1
Tissue healing	No representation	Wound healing	-
		Hemostasis	-
		Blood coagulation	-
		Regulation of body fluid levels	-
		Platelet activation	-
		Complement and coagulation cascades	-

Table 5

Hip fractures in patients day 7 vs. 1 HF(7vs1)				
Gene-ID	log FC	Adj. P. Val (BH)	Gene list	Gene function
210512_s_at	-2,01066	0,00038	VEGFA	Angiogenesis
202337_at	-0,70716	0,00049	PMF1	Osteogenesis
205207_at	-0,59814	0,017	IL6	Inflammation
209201_x_at	-1,14702	0,027	CXCR4	Stem cell attraction
203085_s_at	0,642476	0,042	TGFB1	Osteogenesis
Hip fracture in patients day 42 vs. 1 HF(42vs1)				
Gene-ID	log FC	adj. P. Val (BH)	Gene list	Gene function
210512_s_at	-2,10542	0,00022	VEGFA	Angiogenesis
202337_at	-0,59411	0,0028	PMF1	Osteogenesis
205207_at	-0,61017	0,014	IL6	Inflammation
209201_x_at	-1,28274	0,017	CXCR4	Stem cell acttraction

Figure 1:



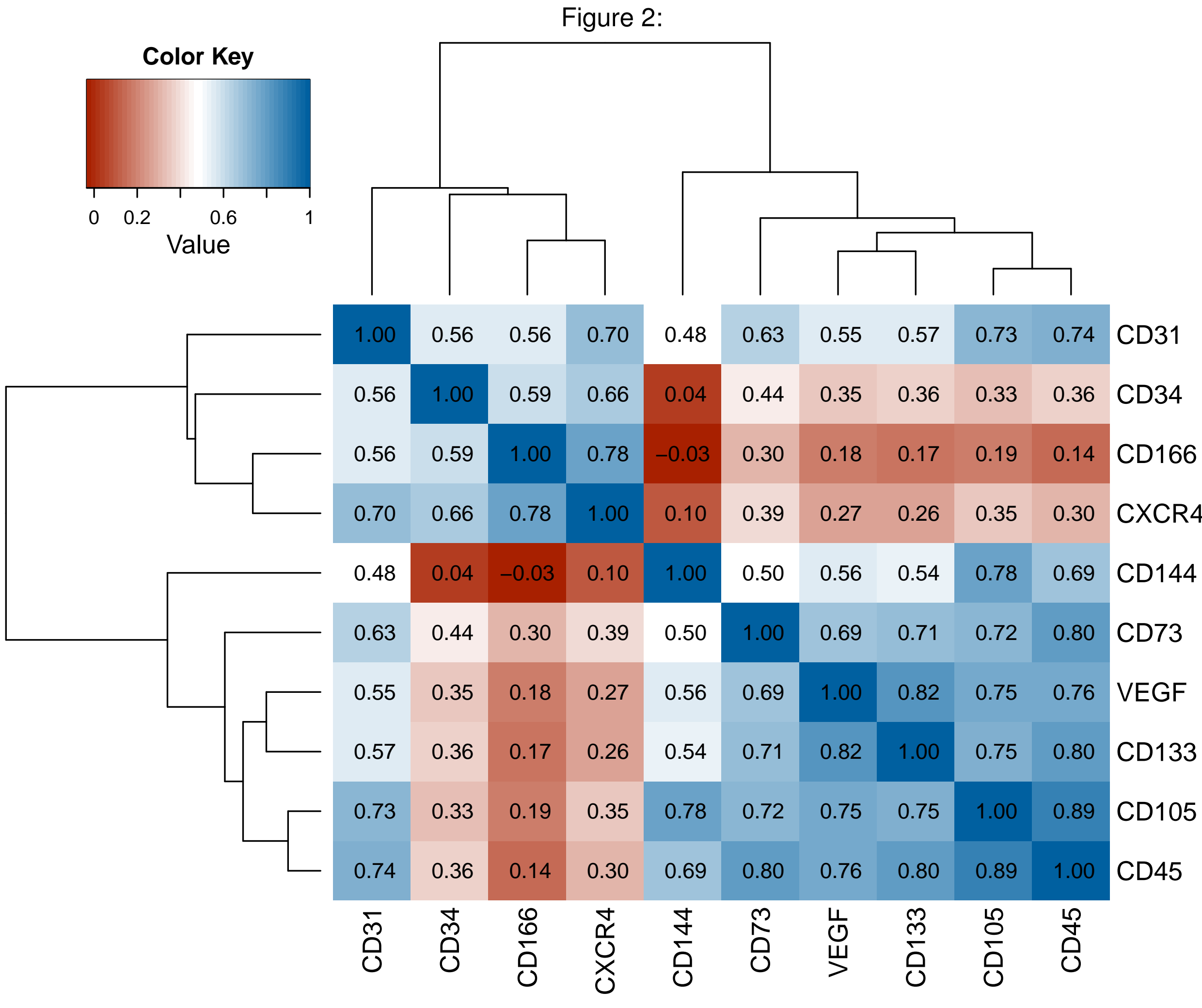
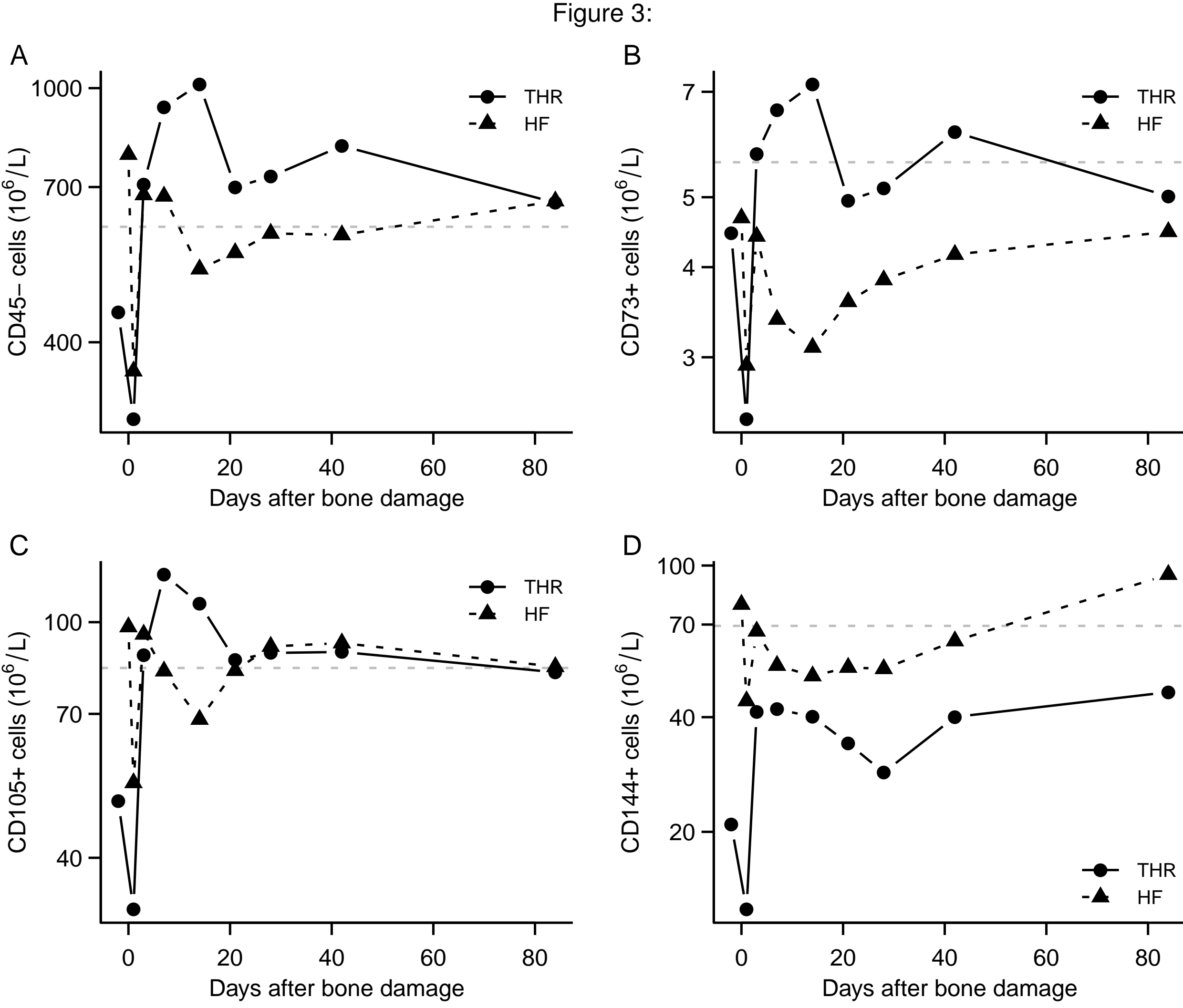


Figure 2:
Color Key
0
0.2
0.6
1
Value
CD31
CD34
CD166
CXCR4
CD144
CD73
VEGF
CD133
CD105
CD45
1.00 0.56 0.56 0.70 0.48 0.63 0.55 0.57 0.73 0.74
0.56 1.00 0.59 0.66 0.04 0.44 0.35 0.36 0.33 0.36
0.56 0.59 1.00 0.78 −0.03 0.30 0.18 0.17 0.19 0.14
0.70 0.66 0.78 1.00 0.10 0.39 0.27 0.26 0.35 0.30
0.48 0.04 −0.03 0.10 1.00 0.50 0.56 0.54 0.78 0.69
0.63 0.44 0.30 0.39 0.50 1.00 0.69 0.71 0.72 0.80
0.55 0.35 0.18 0.27 0.56 0.69 1.00 0.82 0.75 0.76
0.57 0.36 0.17 0.26 0.54 0.71 0.82 1.00 0.75 0.80
0.73 0.33 0.19 0.35 0.78 0.72 0.75 0.75 1.00 0.89
0.74 0.36 0.14 0.30 0.69 0.80 0.76 0.80 0.89 1.00

Figure 3:



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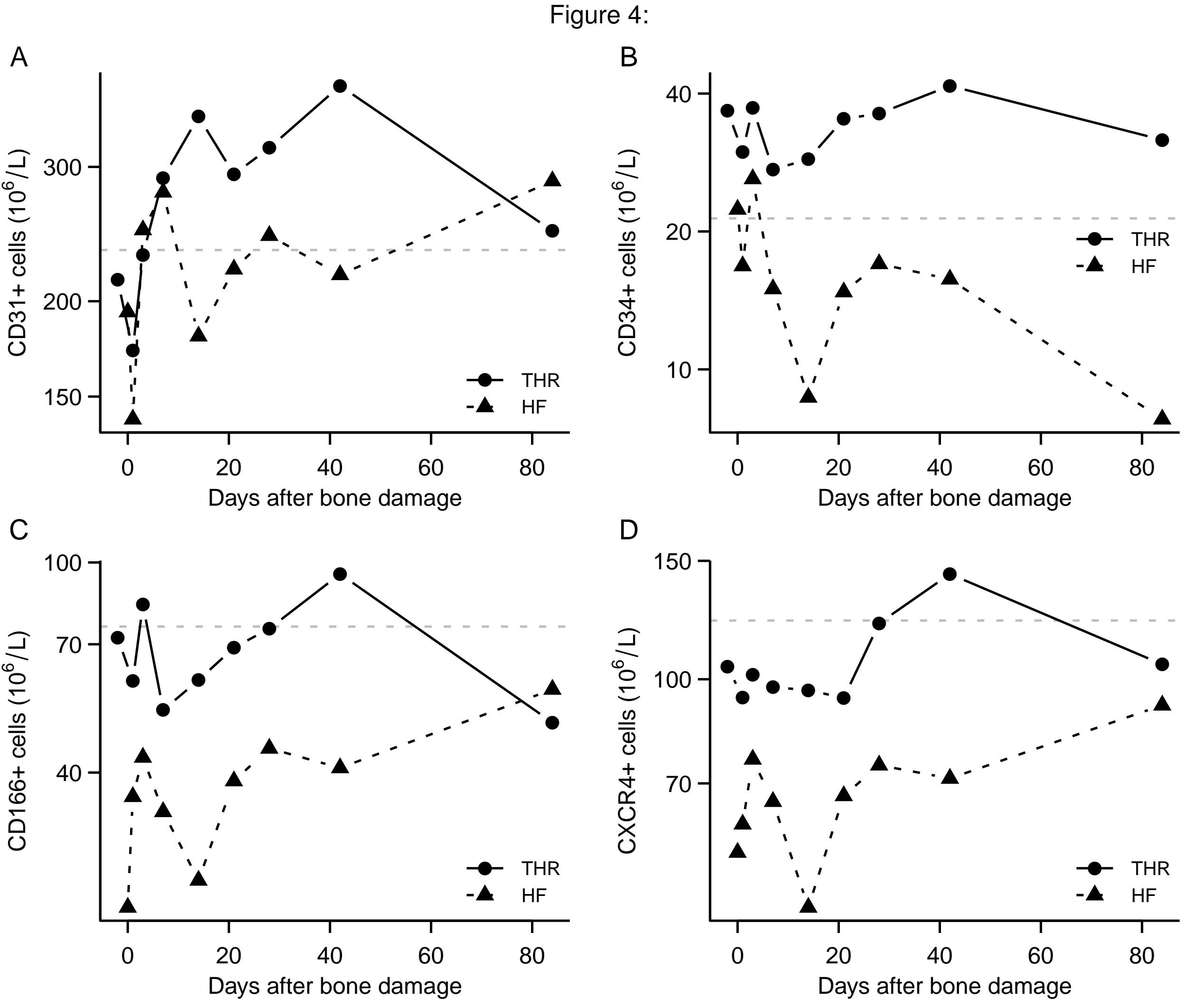

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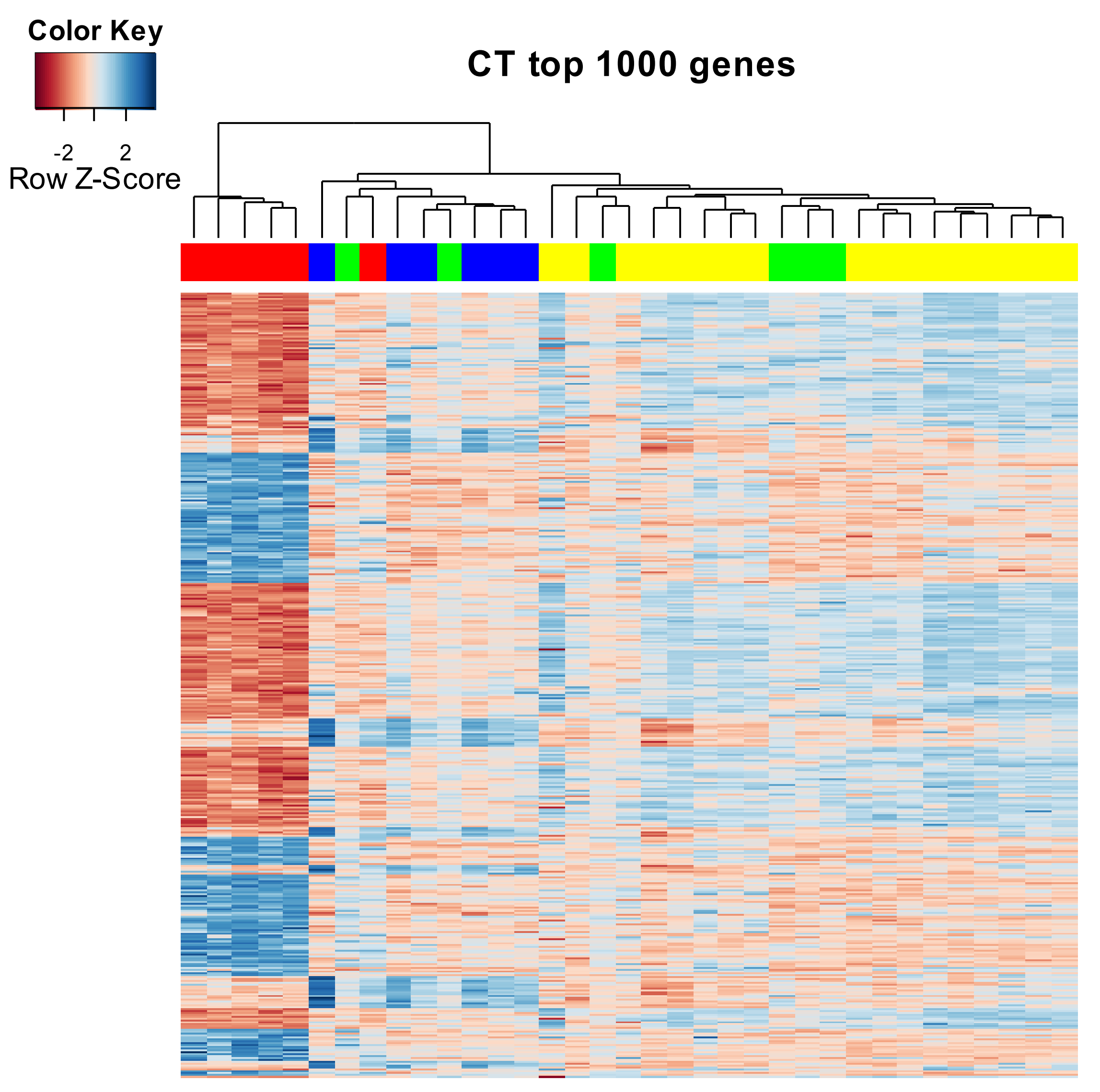

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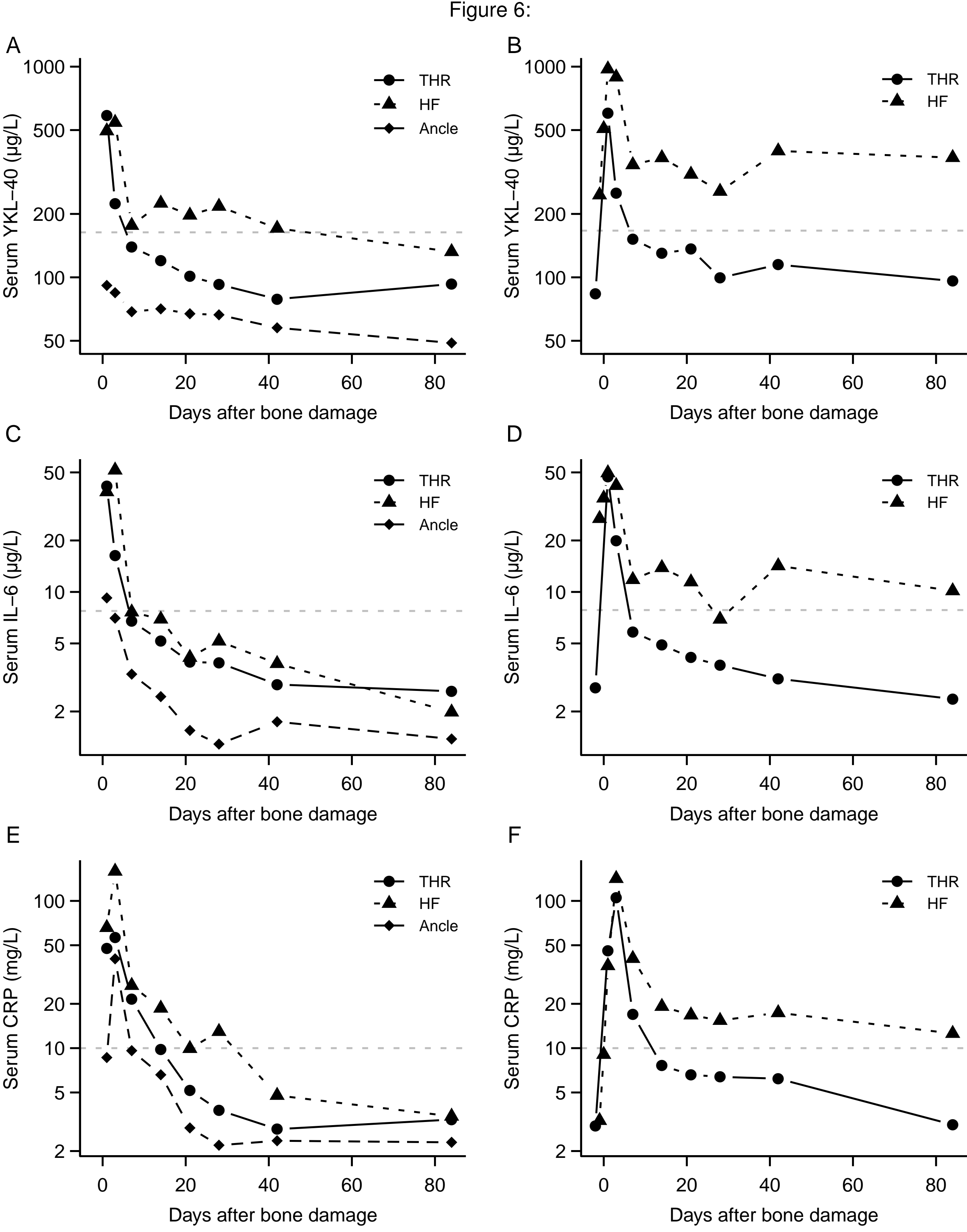

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som indgår i afhandlingen:__

Analysis of circulating mesenchymal progenitor cells and selected cytokines during bone regeneration

indleveret til forsvar af doktorgraden / ph.d.-graden (understreg det gældende).

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25/1-2010	Bo Sandergaard Olsen	Overlæge PhD	[signature]

Erklæring om de enkelte elementer.	Omfang (A,B,C)
1. Formulering i idéfasen af den basale videnskabelige problemstilling ud fra teoretiske spørgsmål, der ønskes afklaret, herunder sammenfatning af problemstillingen til spørgsmål, der skønnes at kunne besvares gennem udførelsen af analyser respektiv konkrete forsøg eller undersøgelser.	A
2. Planlægning af forsøgene/analyser og udformning af undersøgelsesmetodikken på en sådan måde at de under 1 stillede spørgsmål med rimelighed kan forventes besvaret, herunder metodevalg og selvstændig metodeudvikling.	B
3. Involvering i analysearbejdet respektiv den konkrete forsøgsvirksomhed/undersøgelse.	A
4. Præsentation og fortolkning og diskussion af de i artikelform opnåede resultater.	B

Medforfatternes underskrifter.

Dato	Navn	Titel	Underskrift
22/1 10	GUNNAR S. LAUSTEN	OVERLÆGE	[signature]

1. Formulering i idéfasen af den basale videnskabelige problemstilling ud fra teoretiske spørgsmål, der ønskes afklaret, herunder sammenfatning af problemstillingen til spørgsmål, der skønnes at kunne besvares gennem udførelsen af analyser respektiv konkrete forsøg eller undersøgelser.	A
2. Planlægning af forsøgene/analyser og udformning af undersøgelsesmetodikken på en sådan måde at de under 1 stillede spørgsmål med rimelighed kan forventes besvaret, herunder metodevalg og selvstændig metodeudvikling.	B
3. Involvering i analysearbejdet respektiv den konkrete forsøgsvirksomhed/undersøgelse.	A
4. Præsentation og fortolkning og diskussion af de i artikelform opnåede resultater.	B

Medforfatternes underskrifter.

Dato	Navn	Titel	Underskrift
21/1-2010	JENS KASTRUP	OVERLÆGE DR.MED Klinisk forsk. lektor	[signature]

Erklæring om de enkelte elementer.	Omfang (A,B,C)
1. Formulering i idéfasen af den basale videnskabelige problemstilling ud fra teoretiske spørgsmål, der ønskes afklaret, herunder sammenfatning af problemstillingen til spørgsmål, der skønnes at kunne besvares gennem udførelsen af analyser respektiv konkrete forsøg eller undersøgelser.	A
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3. Involvering i analysearbejdet respektiv den konkrete forsøgsvirksomhed/undersøgelse.	A
4. Præsentation og fortolkning og diskussion af de i artikelform opnåede resultater.	B

Medforfatternes underskrifter.

Dato	Navn	Titel	Underskrift
28/1-2010	JULIA SIDENIUS JOHANSEN	PROFESSOR, OVERLÆGE DR.MED	Julia S. Johansen

Medforfattererklæring

I henhold til bekendtgørelsen om doktorgraden § 5, stk. 4 skal der, hvis doktorafhandlingen eller dele heraf er et resultat af gruppearbejde, medsendes erklæring underskrevet af medforfatteren og af forfatteren selv, om omfanget og karakteren af dennes andel i gruppearbejdet.

I henhold til bekendtgørelsen om ph.d.-graden § 2, stk. 3 skal der, såfremt en afhandling bygger på allerede publicerede artikler udarbejdet i samarbejde med andre, følge erklæringer med fra hver af forfatterne om den ph.d.-studerendes andel i arbejdet.

Vejlederen kan optræde som medforfatter hvis kravene i Vancouver-reglerne er opfyldt, jf. Fagligt regelsæt.

Denne medforfattererklæring gælder følgende artikel:_

A clinical model studying circulating cellular and humoral biomarkers involved in bone regeneration following traumatic lesions,

som indgår i afhandlingen:__

Analysis of circulating mesenchymal progenitor cells and selected cytokines during bone regeneration

indleveret til forsvar af doktorgraden / ph.d.-graden (understreg det gældende).

Omfanget af_**Hans Gottlieb's** bidrag *(doktorandens/ph.d.-studerendes navn)*

til artiklen er vurderet ud fra følgende skala.

A. har bidraget til samarbejdet (0-33%).

B. har bidraget i væsentlig omfang (34-66%).

C. har i alt overvejdende grad udført dette arbejde selvstændigt (67-100%).

Erklæring om de enkelte elementer.	Omfang (A,B,C)
1. Formulering i idéfasen af den basale videnskabelige problemstilling ud fra teoretiske spørgsmål, der ønskes afklaret, herunder sammenfatning af problemstillingen til spørgsmål, der skønnes at kunne besvares gennem udførelsen af analyser respektiv konkrete forsøg eller undersøgelser.	A
2. Planlægning af forsøgene/analyser og udformning af undersøgelsesmetodikken på en sådan måde at de under 1 stillede spørgsmål med rimelighed kan forventes besvaret, herunder metodevalg og selvstændig metodeudvikling.	B
3. Involvering i analysearbejdet respektiv den konkrete forsøgsvirksomhed/undersøgelse.	A
4. Præsentation og fortolkning og diskussion af de i artikelform opnåede resultater.	B

Medforfatternes underskrifter.

Dato	Navn	Titel	Underskrift
27-1-2010	Tobias Wirenfeldt Klausen	Statistiker / Datamanager	[signature]

Doktorandens/ph.d.-studerendes underskrift.

Medforfattererklæringen indleveres:

- *til ph.d.-sekretariatet samtidig med afhandlingen til opnåelse af graden*

Det er i begge tilfælde ansøgerens ansvar at fremskaffe medforfattererklæringerne rettidigt. Bedømmelsesudvalget kan ikke behandle sagen, før de foreligger.

Revideret, 17. maj 2004

Accept fra de adspurgte forskere om deltagelse som bedømmere til forsvaret af Hans Gottliebs ph.d.-afhandling

Uddrag af en mail korrespondance mellem Bo Sanderhoff Olsen (Overlæge på Ortopædkirurgisk afdeling T, Herlev hospital og min kliniske vejleder) og Professor, Overlæge Benny Dahl (Rygsektionen på Rigshospitalet).

Kære Hans

hermed accept fra Benny

Bo

<----- Oprindelig email ----->
From: Benny Dahl [bennydahl@gmail.com]
Sent: 5/1/2010 11:52:23 AM
To: Bo Olsen
Subject: Re: CPR nummer og indmelding

Kære Bo,
Vi ses kl. 16.00.
Jo, jeg vil meget gerne være bedømmer på Hans Gottliebs PhD.

Hilsen,

Benny

Uddrag af en mail korrespondance mellem Jens Kastrup (Dr.med., Overlæge på Kardiologisk stamcelle laboratorium, Rigshospitalet og min hovedvejleder) og Ph.d., Overlæge Morten Hanefeld Dziegiel (Blodbanken på Rigshospitalet).

Kære Morten

Tak for tilsagnet.

Hilsen

Jens

Jens Kastrup MD, DMSc, FESC, Associate Professor
Director of Angiogenesis Research Program
Cardiac Catheterization Laboratory 2014,
The Heart Centre, Rigshospitalet University Hospital Copenhagen
9, Blegdamsvej, DK-2100 Copenhagen Ø,
Denmark.
Phone: +45 3545 2819/2817
Fax. +45 3545 2705

-----Morten Dziegiel/HS-Blodbank/DIA/RH/H-S skrev: -----

Til: Jens Kastrup/Hjertemedicin/HJE/RH/H-S@Intranotes
Fra: Morten Dziegiel/HS-Blodbank/DIA/RH/H-S
Dato: 12/03/2009 03:29PM
cc: Hans Erik Johnsen / Region Nordjylland <haej@rn.dk>, Hans Gottlieb - HAGO-Heh@ExchangeKA
Emne: Vedr.: Bedømmelse af phd-afhandling

Kære Jens,
det lyder spændende. Det vil jeg gerne være med til at bedømme.

Med venlig hilsen
Morten Hanefeld Dziegiel, overlæge (MD, PhD)
Blodbanken KI 2034
Rigshospitalet
(Copenhagen University Hospital)
Blegdamsvej 9
DK-2100 Copenhagen
Phone +45 3545 2716
FAX +45 3539 0038
E-mail: dziegiel@rh.regionh.dk

Uddrag af en mail korrespondance mellem Professor, ph.d., forskningsansvarlig Overlæge Søren Overgaard (Ortopædkirurgisk afdeling på Odense universitets hospital, region Syddanmark) og jeg. Korrespondancen er indledt på opfordring af Overlæge, Ph.d., Bo Sanderhoff Olsen (Overlæge på Ortopædkirurgisk afdeling T, Herlev hospital og min kliniske vejleder).

Kære Hans

Den er hermed givet. Jeg vil dog gerne kende det samlede udvalg, hvilket jeg går ud fra at KU vil fremsende.

mvh

Søren Overgaard

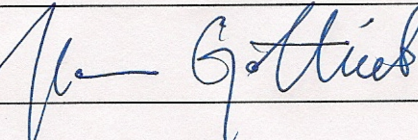
Professor, overlæge, dr. med., forskningsleder Søren Overgaard,
Ortopædkirurgisk Afdeling O
Odense Universitetshospital, Sdr. Boulevard 29, 5000 Odense
Tlf. 6611 3333, direkte 6541 2286, sekretær 6541 3889, Fax 6614 2145
e-mail: soeren.overgaard@ouh.regionsyddanmark.dk
Web: www.sdu.dk/ki/ortopaedkirurgi

Søren Overgaard, Professor, Head of Research, MD, DmSc,
Department of Orthopaedics and Traumatology
Odense University Hospital, Sdr. Boulevard 29, DK-5000 Odense C, Denmark
phone +45 6541 2286, secretary +45 6541 3889, fax +45 6614 2145
e-mail: soeren.overgaard@ouh.regionsyddanmark.dk
Web: www.sdu.dk/ki/ortopaedkirurgi

>>> "Hans Gottlieb" <hago@heh.regionh.dk> 21-01-10 11:01 >>>
Kære Professor Søren Overgaard

Bo Sanderhoff Olsen har fortalt at du har accepteret at indgå i bedømmelsesudvalget til evaluering af min ph.d.-afhandling. Dette er jeg naturligvis meget taknemlig for, men har brug for en skriftlig accept på dette i form af en mail, som jeg kan videresende til ph.d.-sekretariatet med accept fra de øvrige deltagere i dette udvalg.

Venlig hilsen Hans Gottlieb
Ph.d.-studerende
Ortopædkirurgisk afdeling
Herlev hospital

~~Doktorandens~~/ph.d.-studerendes underskrift. 

Medforfattererklæringen indleveres:

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Det er i begge tilfælde ansøgerens ansvar at fremskaffe medforfattererklæringerne rettidigt. Bedømmelsesudvalget kan ikke behandle sagen, før de foreligger.

Revideret, 17. maj 2004