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A Clinical Study of Circulating Cellular and Humoral Biomarkers Involved in Bone Regeneration Following Traumatic Lesions

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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 study of circulating cellular and humoral variables by a time dependent multiparametric approach following traumatic lesions.

Materials and Methods: Two prospective cohorts of 50 patients, with ankle- or hip fracture (cohort 1) or planned hip replacements (cohort 2) were studied as was age matched healthy controls. Blood samples were timely collected during the post traumatic period and analysed for i) non-haematopoietic ($CD45^{neg}$) mesenchymal subsets by multiparametric flow cytometry (MFC), ii) global gene expression profiling (GEP) by micro array and iii) serum inflammatory markers including the growth factor YKL-40 by immunoassays (ELISA). Integrated analyses were performed to identify cellular and humoral patterns with potential impact on tissue regeneration.

Results: Posttraumatic levels of circulating white blood cells, immature progenitor subsets and platelets as well as YKL-40, IL-6 and CRP varied biphasic and correlated to type of traumas.

Analytic MFC identified two minor $CD45^{neg}$ blood compartments which simultaneously expressed varying degrees of CD105, CD133, CD73, VEGF-R, CD144, or CD31, CD34, CD166, CXCR4, respectively, supporting that mesenchymal subsets are involved in fracture healing.

Analysis by microarray identified posttraumatic significant changes in gene expression of specific genes with known relation to inflammation, bone regeneration and angiogenesis in circulating mononuclear cells (MNC).

ELISA quantitation of YKL-40 revealed posttraumatic changes higher in hip traumas compared to patients with ankle fractures (MNC: p=0.0006; YKL-40: p=0.0004). YKL-40 also correlated to the type of bone trauma, documented by different levels in patients with planned surgical hip replacements and traumatic hip fractures (p=0.005).

Conclusions: The present study describes a posttraumatic time dependent cellular and humoral response after planned hip replacements, ankle and hip fractures. The data identify and enumerate potential mesenchymal progenitor cells supporting a regenerative role. Finally the analysis documented a correlation between the growth factor YKL-40 and bone traumas separating it from IL-6 and CRP. These observations can be used to future identification, isolation and characterization of circulating cells with impact in bone regeneration.

Keywords: Biomarkers; Bone regeneration; Fracture; Gene expression; Mesenchymal progenitor cells; YKL-40

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 osteoprogenitors in the periosteum undergo intramembranous ossification, which is the formation of new bone. During this regenerative stage the fracture site is divided into a central cartilage containing zone and a peripheral bone containing zone. The zone division of the callus is partly due to the distance from the vascularised fracture ends and periosteum to the mesenchymal progenitor cells, which influence whether these cells differentiate into bone or cartilage through changes in pH-levels and oxygen tension. 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,

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OPG and TNFα also regulate the later endochondral bone remodeling [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, [20], arthritic chondrocytes, inflammatory cells and endothelial cells. The full biological functions of YKL-40 are still 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 after osteosynthesis [40].

The observation that solid tissues are colonized by organ-specific circulating blood cells suggests that organ specific tissue regeneration and repair may be feasible if we learn to 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 2005 to 2007 with an ankle- or hip fracture (cohort 1) or a planned hip replacement due to osteoarthritis (cohort 2). The hip fractures were all located at the femoral neck and were all operated with the same technique, which in short consists of a thick stabilizing screw in the femoral neck, supported by a plate on the lateral side of the femur. The material is placed through a lateral sharp approach through the skin. Patients with planned hip replacements all had osteoarthritis and all were operated in the same way with a cement fixated total hip prosthesis, which was placed by access through the gluteus muscle.

Patients with multiple fractures, severe cardiopulmonary disease, and surgery within 6 months or malignancy were excluded. Demographical data were registered from medical records included age, sex, type of bone damage, complications and co-morbidity (Table 1).

The study was prospectively planned and conducted but extended due to initial experiences including: i) failed attempts to perform flow cytometry analyses of surface markers of frozen cells (data not shown), ii) lack of plasma samples within the first 24 hours following fracture, iii) no presurgical baseline value before planned hip replacements, iv) minor cellular responses following ankle fractures.

Patients in cohort 2 were consecutively included under the same criteria as patients in cohort 1, but with additional samples 3-5 and 12-15 hours after fracture and a presurgery sample taken before planned hip replacements.

Of 37 patients included in cohort 1, five patients were later excluded (1 patient with ankle fracture refused to participate, 2 patients with hip fractures 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 religion, 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, severe cardiovascular morbidity or known malignancy within 6 months of inclusion.

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 before operation, 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 blood in one 10 ml Serum Sep Clot Activator glass.

Between ½-3 hours after vein puncture stabilized blood samples

Bone trauma	No. of pt. in./ex./ completed	Age/ years range	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

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

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 $NaH_2PO_4 \cdot H_2O$, 6.5 mM $NA_2HPO_4 \cdot 2H_2O$, 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 from patients in cohort 1 and 2 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]. Fresh isolated MNCs from patients in cohort 2 were analysed by multi parametric flow cytometry as described below.

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 expected 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. All the antibodies were conjugated to the cells simultaneously.

The remaining cell pellet was mixed with 350µl FACS-PBS + 10%NCS + EDTA and applied for analysis by a four colour flow cytometry (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

Associated cell population	Cluster of differentiation	Molecule	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	VEGFR-2	PE
Injured and hypoxic tissue	CD184	CXCR4	PE
Negative control	IgG1(Mouse)		all

Table 2: The panel of selected CD specific antibodies used to identify and enumerate subpopulations by multiparametric flow cytometry (MFC).

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 array Quality Metrics [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 heat maps. We used the heamap.2 function from the R-package gplots. The hierarchical cluster analysis indicated in these heat maps 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 Immuno Sorbent 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 Dakota Cytomation, 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 YKL-40 values of the healthy controls [35]. 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 $CD45^{neg}$ MNCs with an initial reduction followed by a rise day 1-2 and an overshoot in the following week (Figure 1A and B).

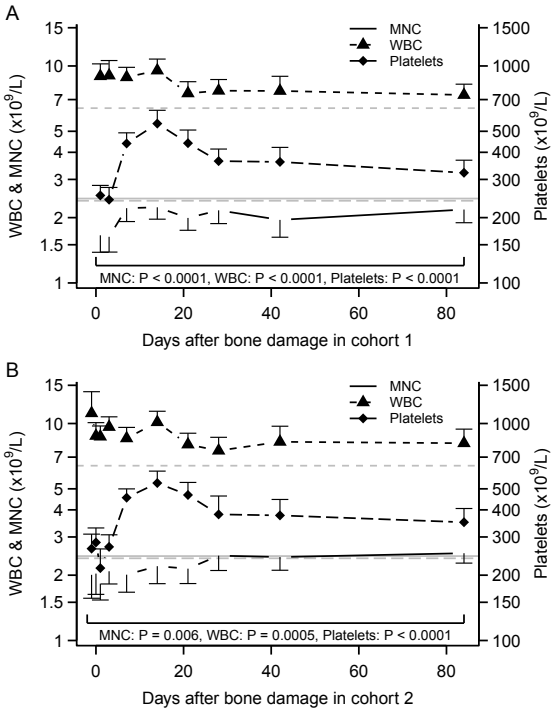

Figure 1: Quantification of circulating leucocytes (WBC), mononuclear cells (MNC) and platelets in cohort 1 are shown in figure 1A and cohort 2 is shown in figure 1B. Healthy controls had an average age of 71 years (63-81) and a mean number of circulating leucocytes of 6.53 x 10^9/L (4.1 x 10^9/L–8.8 x 10^9/L), MNC of 2.53 x 10^9/L (1.19 x 10^9/L-3.57 x 10^9/L). Levels for healthy controls are grey lines in the figures.

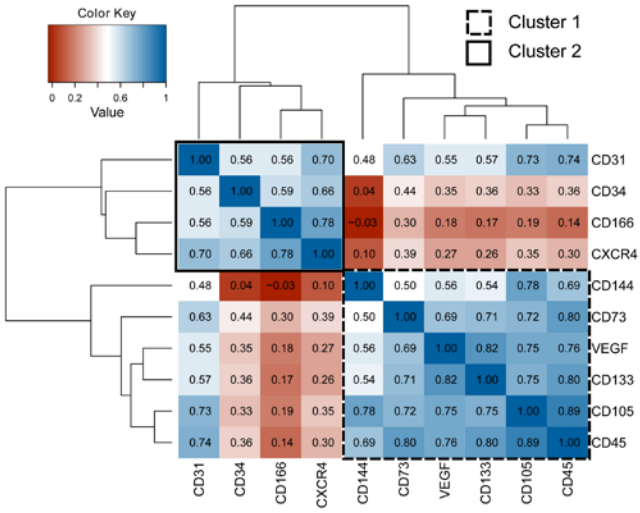

Figure 2: The $CD45^{neg}$ MNCs clusters in two subpopulations according to the changing intensities of the specific surface markers during bone regeneration. The underlined and dotted squares illustrate the division of the surface markers in respectively cluster 1 and cluster 2 according to their internal correlations. The numbers in the squares are the correlation coefficients, which are also explained in the colour key.

Enumeration of non-haematopoietic subpopulations in circulation

MFC analyses of circulating $CD45^{neg}$ cells in all patients with surgical or traumatic bone traumas revealed the phenotype of two homogeneous clusters during bone regeneration, according to intensities of the specific surface markers given in Figure 2. The underlined and dotted squares illustrate two time correlated clusters (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 vascular outflow from the circulating blood into reactive areas. The cells 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).

Micro array gene expression in circulating MNC

With a false discovery rate (FDR) at 0.5%, 1462 genes changed expressions levels in MNCs from day 1 to 7 after hip fractures. A total of 1456 genes changed expression levels when comparing day 1 with day 42 after hip fracture.

A heat map (Figure 5) including the most varying genes in MNCs from hip fractures and healthy controls illustrate how the expression changes during the first 24 hours after the fracture and then normalizes towards the pre-fracture homeostatic equivalence, during the initial 42 days after the trauma. Even though the numbers of genes that changed expression level from day 1 to day 7 and 42 respectively were almost similar, the functional clustering of the up- or down regulated genes

differed (Table 3 and 4). Gene clusters of inflammation, cellular activity and cellular stress played a more important role day 7, than day 42. This is 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 related sub-clusters. The EASE-scores (enrichment-score) of 1.3 is equivalent to a non-log scale 0.05, meaning that attention should be given towards gene-clusters with EASE-scores >1.3. The key-cluster of "Tissue healing" was not significantly present at day 42 compared to day 7 (Table 3 and 4).

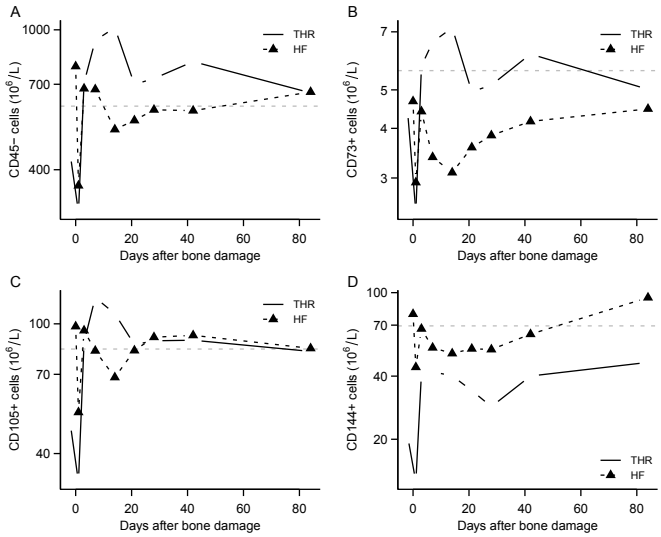

Figure 3: Show changes during bone regeneration in CD45^{neg} MNC (A), CD73^{pos}, CD45^{neg} MNC (B), CD105^{pos}, CD45^{neg} MNC (C), CD144^{pos} CD45neg MNC (D) (Cluster 1). The initial drop is underlined by p-values. The grey line shows the level of the specific surface marker in healthy subjects.

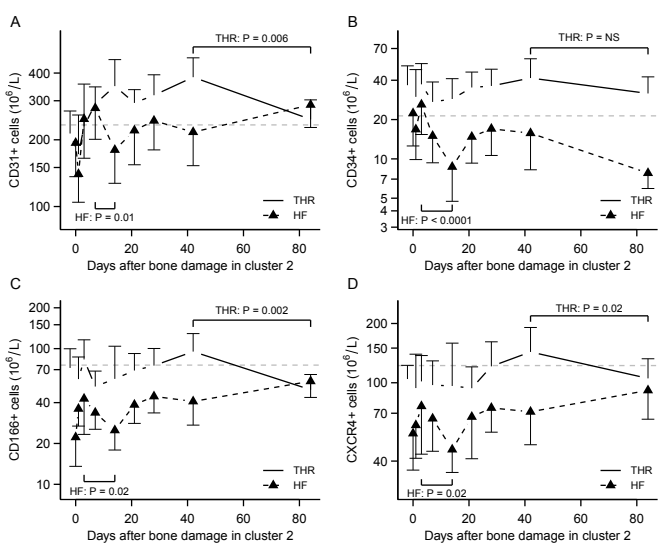

Figure 4: Show changes during bone regeneration in CD31^{pos}, CD45^{neg} MNC (A), CD34 ^{pos}, CD45^{neg} MNC (B), CD166 ^{pos}, CD45^{neg} MNC (C) and CXCR4^{pos}, CD45^{neg} MNC (D) (Cluster 2). The decline at day 14 of all the surface markers on cells in cluster 2, which are only seen in patients with hip fractures, is documented by p-values in the figure. The grey line shows the level of the specific surface marker in healthy subjects.

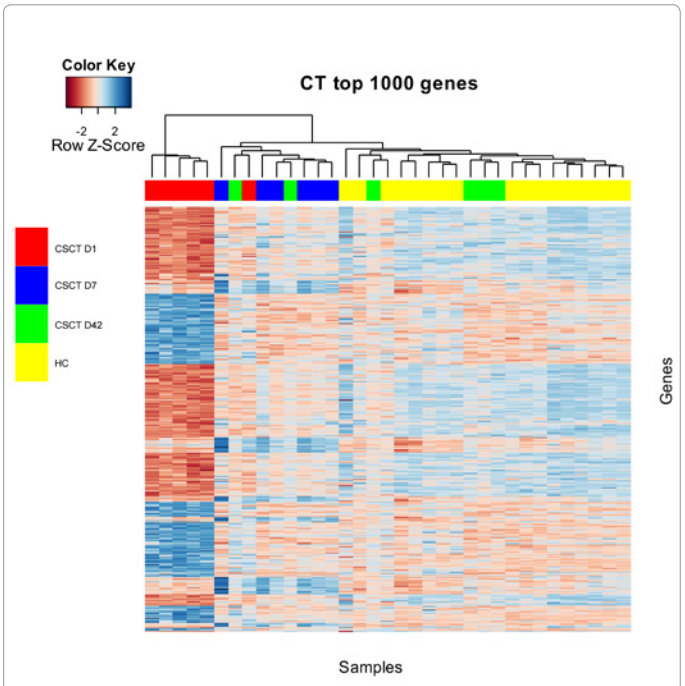

Figure 5: The heat map shows the standardised values of the 1000 most time-varying genes. Red bars mean an up regulation of the gene and blue bars mean a down regulation of the gene. The genes are row-wise sorted by a cluster algorithm according to their similarity with other genes. Likewise are the samples column-wise sorted by a cluster algorithm according to their similarity with other samples. The coloured squares between the dendrogram and the heat map indicate the time point of the collection: Columns under red squares are taken 3-5 hours after fracture (D1), columns under blue squares are taken at day 7 after fracture, columns under green squares are taken at day 42 after fracture and columns under yellow squares are from healthy subjects. The genetic expression changes during bone regeneration from very aberrant at 3-5 hours after fracture, towards almost normal at day 42.

Serum biomarkers following fracture and during regeneration

Patients with hip fractures or planned hip replacements had higher serum YKL-40 concentrations 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. YKL-40 increased after trauma in patients with hip fractures (P=0.0001) and after surgery in patients who got planned hip replacements (P<0.0001) (Figure 6A and 8B).

Serum IL-6 rose during day 1 after planned surgery or fracture (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 8D). IL-6 were higher at day 1 in patients with hip fractures or planned hip replacements, than in patients with ankle fractures (P<0.0001). This indicates a relation between IL-6 and the magnitude of the traumatized bone (Figure 6C). There were no differences in IL-6 levels in patients with hip fractures or planned hip replacements, during early bone regeneration between day 14 and 42 (P=0.4), which indicates that IL-6 does not differ according to the type of bone trauma, in contrast to YKL-40..

Changes in s-CRP are known to quantify ongoing inflammation as IL-6. CRP rose posttraumatic (P<0.0001), but with a significant difference between patients with traumatic hip fractures or planned hip replacement and patients with ankle fractures (P=0.0021) (Figure 6E). Posttraumatic changes in s-CRP were considerable in patients with hip fractures (P<0.0001) and patients with planned hip replacements

Key cluster	EASE-score	Sub-cluster	P-value (Benjamini)
Transcription binding	5.7	Transcription factor binding	4.4E-6
		Transcription cofactor activity	7.5E-4
Apoptosis	4.86	Regulation of apoptosis	1.7E-5
		Cell development	1.6E-2
		Cell differentiation	3.3E-1*
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 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

* correlated to “cell development” (κ=0.87)

Table 3: Key- and sub-clusters of genes associated to the ongoing posttraumatic inflammation or bone regeneration. The genes are differentially expressed at day 1 and day 7 in patients with hip fractures.

Key cluster	EASE-score	Sub-cluster	P-value (Benjamini)
Transcription binding	3.25	Transcription factor binding	3.0E-2
		Transcription 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 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	None	Wound healing	-
		Hemostasis	-
		Blood coagulation	-
		Regulation of body fluid levels	-
		Platelet activation	-
		Complement and coagulation cascades	-

Table 4: Key- and sub-clusters of genes associated to the ongoing posttraumatic inflammation or bone regeneration. The genes are differentially expressed at day 1 and day 42 in patients with hip fractures.

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 attraction

Table 5: Identification of Benchmark-genes expressed in blood MNC from hip fractured patients.

(P<0.0001) (Figure 6E and 8F). As seen for IL-6, did CRP not differ in patients with hip fractures or in patients with planned hip replacements, indicating that CRP is unspecific according to the type of hip trauma (P=0.13 and p=0.08).

Discussion

Patients with traumatic hip or ankle fractures regenerate through intra-membranous and endochondral ossification, in contrast to patients with total hip replacements due to osteoarthritis, who have their implants fixated through cementation with a minor amount of bone formation and risk of aseptic loosening of the prosthesis [47-49]. It is the *hypothesis* of this research project that extended knowledge of fracture pathophysiology may allow us to predict the quality of repair mechanisms and risk of side effect of therapy.

The present knowledge of bone repair mechanisms and the pathophysiology of fracture healing are mainly based on animal studies. Differences in the anatomy and metabolism of animals and humans are limitations in the transfer of experimental data from animal models into clinical situations. Therefore, the present research project addresses a human clinical model with special emphasis on the time dependent involvement of circulating mesenchymal progenitor cells and humoral biomarkers in bone repair [4,5,7,22].

The *specific aims* of this study was to measure the initial and later changes of the number of specific compartments of mononuclear cells and levels of IL-6, CRP and YKL-40 in peripheral blood following bone injuries in two prospective cohorts including 50 patients, with ankle- or hip fracture (cohort 1) or planned hip replacements (cohort 2) compared to age matched healthy controls.

The work plan included timely collected blood sampling during the post traumatic period and analysis for i) non-haematopoietic ($CD45^{neg}$) mesenchymal subsets by multiparametric flow cytometry (MFC), ii) global gene expression profiling (GEP) by micro array and iii) serum inflammatory markers including the growth factor YKL-40 by immunoassays (ELISA). Subsequent integrated analyses were performed to identify cellular and humoral patterns with potential impact on tissue regeneration – for future isolation and characterization.

First, *multiparametric flow cytometry analyses* of circulating MNCs revealed two homogeneous clusters of $CD45^{neg}$ MNCs (Figure 2). The two clusters were defined by similar changes over time for specific surface makers. One cluster consisted of $CD45^{neg}$ MNCs with presence of the surface markers CD73, CD105 and CD144, which are known to be associated with mesenchymal progenitor cells [8,50,51]. The second cluster consisted of $CD45^{neg}$ MNCs with presence of the surface markers CD31, CD34, CD166 and CXCR4. The surface markers CD31 and CD34 are associated with mature endothelial- and endothelial progenitor cells, which are involved in neo-angiogenesis [52,53]. Reperfusion of the ischemic fracture haematoma is of utmost importance for of blood born mesenchymal progenitor cells. CD166 is associated to mesenchymal progenitor cells and cell adhesion [54,55]. CXCR4 is a chemokine receptor present on MNCs, which are drawn towards traumatized or ischemic tissue, because of the secretion of SDF-1 (stromal cell derived factor 1) from such tissues[19,56]. The presence of specific surface markers on cells within the same cluster indicates a similar and related role in regeneration of traumatized- and ischemic bone.

Second, *extended microarray analyses* of the posttraumatic circulating MNCs identified 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, which goes 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 of circulating MNCs in actual bone regeneration, inflammation and neo-angiogenesis 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].

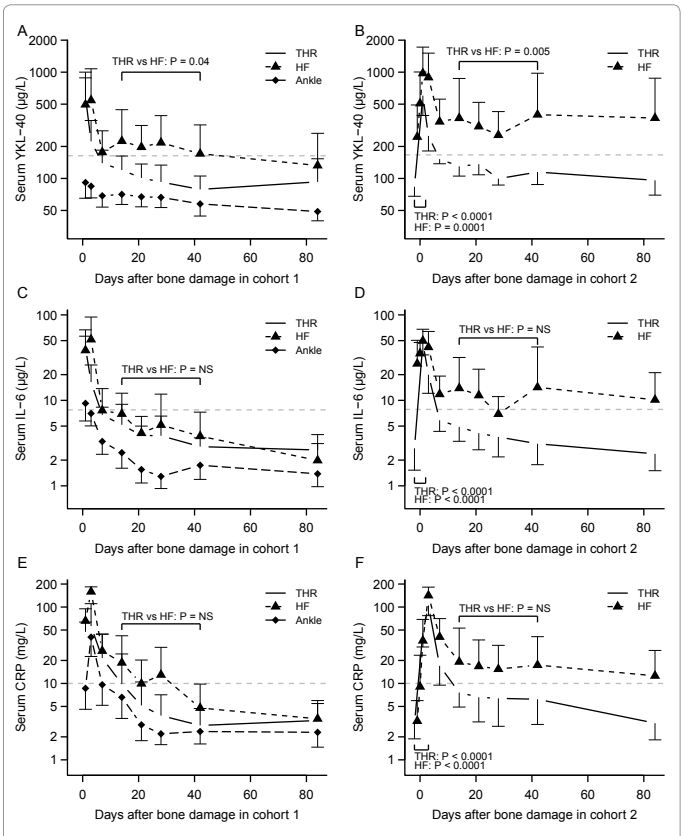

Figure 6: Figure 6A, 6C and 6E shows changes of YKL-40, IL-6 and CRP in patients in cohort 1. Figure 6B, 6D and 6F shows changes of YKL-40, IL-6 and CRP in patients in cohort 2. Dotted grey lines mark upper boundaries of normal levels of the cytokines. The initial rise in all the cytokines within the first day in patients with hip fractures are documented by p-values for patients in cohort 2 at the bottom of the figure. The elevated levels of YKL-40 from day 14-42 in patients with hip fractures compared to patients with total hip replacements in cohort 1 and 2 are documented by p-values at the top of figures 6A and 6B.

Third, *the humoral inflammatory response* that we quantified through serum levels of YKL-40, IL-6 and CRP, correlated significantly to the magnitude of traumatized bone, documented by larger posttraumatic changes in YKL-40, IL-6 and CRP in patients with hip fractures or planned hip replacements than in patients with ankle fractures. The higher level of YKL-40 during initial bone formation from day 14 to 42 in patients with hip fractures than in patients with planned hip replacements may indicate that YKL-40 is a quantitative marker of endochondral ossification, independent of age. YKL-40, in contrast to IL-6 and CRP, correlated to both the type and magnitude of bone traumas, which again underlines a more central role involvement in actual bone formation, which separates it from the other inflammatory markers. 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].

Though IL-6 has been shown to be involved in bone mineralization and remodelling in rats and that elevation on IL-6, also in rats, has an adverse effect on bone regeneration, we did not see any correlation between type of hip trauma and IL-6 [21,24-26].

In conclusion, this report describes the nature and concentration of posttraumatic circulating progenitor cell compartments, identifies reactive genes and functional clusters of these expressed in circulating mononuclear cells and involved inflammatory and regenerative biomarkers YKL-40, IL-6, and CRP during regeneration of surgical and traumatic bone traumas. This clinical in vivo model will be used to identify, isolate and characterize the potential mesenchymal progenitor cell compartment, analyses of specific gene expression for future functional preclinical studies.

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