

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

## The Role of Circulating Bone Cell Precursors in Fracture Healing

### **This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/76080> since 2016-10-25T14:42:05Z

*Published version:*

DOI:10.1007/s00223-010-9362-3

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



# UNIVERSITÀ DEGLI STUDI DI TORINO

***This is an author version of the contribution published on:***

*Questa è la versione dell'autore dell'opera:*

*The role of circulating bone cell precursors in fracture healing.*

*D'Amelio P, Cristofaro MA, Grimaldi A, Ravazzoli M, Pluviano F, Grosso E,*

*Pescarmona GP, Isaia GC.*

*Calcif Tissue Int. 2010 Jun;86(6):463-9. doi: 10.1007/s00223-010-9362-3..*

***.The definitive version is available at:***

*La versione definitiva è disponibile alla URL:*

*[<http://link.springer.com/article/10.1007%2Fs00223-010-9362-3>]*

## **The role of circulating bone cell precursors in fracture healing.**

Patrizia D'Amelio<sup>1</sup>, Maria Angela Cristofaro<sup>1</sup>, Anastasia Grimaldi<sup>1</sup>, Marco Ravazzoli<sup>1</sup>, Fernanda Pluviano<sup>2</sup>, Elena Grosso<sup>3</sup>, Gian Piero Pescarmona<sup>4-5</sup>, Giovanni Carlo Isaia<sup>1</sup>.

<sup>1</sup> Department of Surgical and Medical Disciplines Section of Gerontology, University of Torino-Italy

<sup>2</sup> Department of Imaging, Faculty of Medicine, University of Torino, Italy

<sup>3</sup> Department of Orthopaedics, Traumatology - CTO-CRF-ICORMA Hospitals- Torino-Italy

<sup>4</sup> Center for Experimental Research and Medical Studies (CERMS)- San Giovanni Battista Hospital – Torino-Italy

<sup>5</sup> Department of Genetics, Biology and Biochemistry -University of Torino – Italy

**Running title:** Fracture healing

**Corresponding author:** D'Amelio Patrizia MD, PhD

Department of Surgical and Medical Disciplines Section of Gerontology,

Corso Bramante 88/90, 10126 Torino, Italy.

Tel: +390116335533-Fax: +390116636033, e-mail: [patrizia.damelio@unito.it](mailto:patrizia.damelio@unito.it)

## **ABSTRACT.**

**Purpose:** Fracture healing is a complex process that involves several cell types; as a previous report suggested an increase in osteoblasts (OB) precursors in peripheral blood during this process, this paper examines the role of circulating bone cell precursors in this process in the light of a prior suggestion that osteoblast (OB) precursors are increased.

**Methods:** Nine healthy men under 60 with traumatic fractures were enrolled. The following parameters: circulating OB precursors (Osteocalcin+/Alkaline Phosphatase+/CD15- cells and osteoclast precursors (CD14+/CD11b+/Vitronectin Receptor+ cells) were measured by flow cytometry, bone formation markers and TGF $\beta$ 1 by ELISA and PTH by RIA in serum on arrival in the emergency department (baseline) and 15 days after fracture.

**Results:** Bone cell precursors behaved differently during healing. TGF $\beta$ 1 was inversely correlated with OB number, but increased their degree of maturation at baseline. Bone formation markers and TGF $\beta$ 1 were increased after fracture, whereas PTH was decreased. The TGF $\beta$ 1 increase was directly correlated with age, whereas age was not correlated with the precursors.

**Conclusions:** We confirm the role of TGF $\beta$ 1 in fracture healing; and its possible role in the control of pre-OB homeostasis. There was no variation in circulating precursor cells during healing, though the increase in TGF $\beta$ 1 may suggest increased pre-OB maturation and homing to the injured site.

**Keywords:** osteoblast precursors; osteoclast precursors, peripheral blood, TGF $\beta$ , fracture healing.

## INTRODUCTION.

Osteoclast lineage cells are present in the peripheral circulation and increased in bone lytic diseases (reviewed in [1]). They are decreased by drugs active on bone turnover such as bisphosphonates [2, 3]. Whether a comparable pool of circulating osteoblast (OB) lineage cells is present and has a role in bone metabolism is uncertain [4]. It has been suggested that osteogenetic cells are present at extremely low concentrations in the human peripheral blood, express typical OB markers such as alkaline phosphatase (AP) and osteocalcin (OC), and form mineralized nodules in vitro and bone tissue in vivo in nude mice [5, 6]. Their physiological role during skeletal growth and fracture repair has also been proposed. Eghbali-Fatourehchi et al [5] observed that cells positive for OC, but not for AP, were over five times more numerous in the circulation of adolescent boys compared to adult men, and that they increased after fracture. Reduction of the circulating OB precursors pool has been described in postmenopausal osteoporosis [7]; however the physiological role of these cells in bone metabolism remains to be clarified.

Fracture healing is still viewed as an unresolved cascade of complex biological events involving intracellular and extracellular molecular signalling for bone induction and conduction [8]. Molecular mechanisms that regulate skeletal tissue formation in utero are repeated during this process [9]. Many local and systemic regulatory factors, including growth and differentiation factors, hormones, cytokines, and extracellular matrix, interact with several cell types, including bone and cartilage-forming primary cells recruited to the fracture site from the bone marrow [10, 11] or the circulation [12]. One of the most studied systemic factors is transforming growth factor  $\beta$  1 (TGF $\beta$ 1), which is a potent chemotactic stimulator of mesenchymal stem cells that enhances the proliferation of OBs precursors and chondrocytes, and may act in recruitment of bone cells in the fractured bone [13]. It induces the production of extracellular bone matrix proteins such as collagen, proteoglycans, osteopontin, osteonectin, and alkaline phosphatase (AP) [14], and regulates different cell types directly involved in bone remodeling and fracture healing [15-17].

A growing body of evidence indicates that intermittent treatment with parathyroid hormone (PTH) (1-34) may promote fracture healing [18], and suggests that endogenous PTH may be equally involved.

This paper assesses the role of circulating bone precursor cells in fracture healing in relation with TGF $\beta$ 1 and PTH in nine males.

## **METHODS.**

### *Patients and bone turnover markers.*

The study was approved by the "Clinical Study Review Committee" of the San Giovanni Battista Hospital, Torino, and all patients signed an informed consent statement prior to recruitment.

Nine healthy men aged 15 to 59 (mean 33, SD 14) with a traumatic fracture (see Table 1) were enrolled. None were on calcium and vitamin D, thyroid hormones, corticosteroids, bisphosphonates, or PTH. The presence of diseases that influence bone metabolism was ruled out on the basis of the medical history and physical examination. Forty ml of peripheral blood were obtained upon arrival in the emergency department (baseline) and 15 days after the fracture.

### *Cell isolation*

Peripheral blood mononuclear cells (PBMCs) were obtained with the Ficoll-Paque method from 30 ml of blood in lithium heparin, as previously described [19]. The serum was frozen at -80°C until the measurements were done.

### *Flow cytometry*

To evaluate the role of circulating osteoclast and OB precursors in fracture healing, we measured them in the PBMCs at baseline and 15 days after fracture. Briefly, osteoclast precursors were evaluated by staining PBMCs with fluorescein (FITC, supplied by B&D) conjugated anti-VNR, phycoerythrin (PE, supplied by B&D) conjugated anti-CD14 and allophycocyanin (APC, supplied by B&D) conjugated anti-CD11b mAb, or with the corresponding isotype control, followed by incubation at 4°C for 30 min as previously described [2, 3, 20]. CD14+/CD11b+/VNR- cells were regarded as early osteoclast precursors [2, 3, 21, 22]; and triple-positive (CD14+/CD11b+/VNR+) cells as osteoclast precursors according to the literature [2, 3, 20, 23-28].

OB precursors were evaluated by staining PBMCs with FITC conjugated anti-CD15 (in order to exclude granulocytes that expressed AP, supplied by e-Bioscience), APC conjugated anti-AP (supplied by R&D System Inc), PE conjugated anti-OC (supplied by R&D System Inc), or with the corresponding isotype control, followed by incubation at 4°C for 30 min as previously described [5]. CD15-/AP+/OC+ cells were regarded as OB precursors according to the literature [5].

Membrane antigen expression was analyzed with the CellQuest software (Becton Dickinson & Co), and displayed as bivariate dot plots or histograms. Each plot depicts the results from 10,000 events representing viable cells gated by cell size and granularity.

### *Cytokine measurement*

ELISA kits were used to measure bone AP (BAP, Instant ELISA; Bender), OC (Instant ELISA; Bender) and TGF  $\beta$ 1 (DuoSet, R&D System Inc). PTH was measured with a radioimmunologic assay (RIA-IRMA-Pantec).

### *Statistics*

The normal distribution of each parameter was determined with Kurtosis, since none were normally distributed; the Wilcoxon-Mann-Whitney test was used to compare values at baseline and 15 days after the fracture. Correlations between parameters were performed by means of Spearman's coefficient or with partial correlation after correction for age. The SPSS 17.0 software package was used to process the data with  $p < 0.05$  as the significance cut-off.

## **RESULTS**

### *Circulating OB and osteoclast precursors do not vary during fracture healing.*

Despite the presence of a clinically substantial callus, no variation was observed in the percentage of circulating OB and osteoclast precursors (Fig. 1 A,B), nor in their degree of maturation as evaluated by the mean fluorescence intensity (MIF) of the molecules studied (data not shown). There was a high individual variability in the bone precursor cells, especially in circulating pre-OB: OC<sup>+</sup> cells increased in 5 patients and OC<sup>+</sup>/AP<sup>+</sup> cells in 3 (Fig. 2).

There were no evident clinical differences between these patients and those who did not display such increases. By contrast, bone formation markers increased in all nine patients during fracture healing, (Fig.3 AB).

There were no correlations between these markers and OB precursors

### *TGF $\beta$ 1 increases during fracture healing.*

Since TGF $\beta$ 1 plays an important role in the local recruitment of osteogenic cells during fracture healing [13], we measured it in serum and found that it increases after fracture (Fig 3 C). As age could influence TGF $\beta$  levels [29, 30], we correlated this parameter with OB precursors after correction for age; TGF $\beta$  was inversely correlated with OB precursors ( $R = -0.77$ ,  $p = 0.25$ ) and directly correlated with the OC MIF ( $R = 0.86$ ,  $p = 0.027$ ): these correlations disappeared during healing. These data suggest that systemic TGF $\beta$ 1 may influence the degree of maturation of OB precursors in physiological condition, while the increase in TGF $\beta$ 1 after fracture may influence

the recruitment of osteogenic cells from the circulation into the fracture site by enhancing their homing ability.

*PTH decreases during fracture healing.*

To assess the role of endogenous PTH in fracture healing, we measured its level at baseline and after fracture: PTH values were always within the physiological range (10-65 pg/mL) and decreased, although not significantly, during healing (Fig.3 D). PTH is inversely correlated with the degree of maturation of OB precursors at baseline, whereas this correlation disappears during healing (Fig.4).

None of the measured parameters correlates with osteoclast precursors (data not shown).

*Age affects TGF $\beta$ 1 and BAP during fracture healing.*

Since fracture healing slows with age and gene expression in callus varies accordingly [29], we evaluated the relationship between age, bone cell precursors and the variation of bone formation markers, TGF $\beta$ 1 and PTH levels. Age was not significantly correlated with bone cell precursors (data not shown). Figure 5 shows that age is directly correlated with the increase in BAP and TGF $\beta$ 1 after fracture, but not with the decrease in PTH.

## **DISCUSSION**

Fracture healing is a complex process that involves a variety of cells, such as hematopoietic cells, platelets, immune system cells, fibroblasts and bone cells. On the strength of a recent suggestion [5] that circulating OB precursors are involved in fracture healing, we evaluated both OB and osteoclast precursors in the peripheral blood with respect to bone formation markers, TGF $\beta$ 1 and PTH at baseline and 15 days after a traumatic fracture. The period of 15 days was chosen because of its clinical significance in the evaluation of callus formation, also because of the previous observation of an increase in OB precursors 2 weeks after fracture [5]. As expected, there was an increase in bone formation markers. There was no significant variation in the number of circulating bone cell precursors, nor was there any correlation between the bone formation markers and OB precursors. Eghbali-Fatourehchi et al. [5], however, observed a striking increase in the percentage of OB precursors two weeks after fracture in 3 healthy men, whereas that these cells may either decrease or increase after fracture. We conclude that there are evidently significant between-subject differences in the extent to which the number of OB precursors is influenced by fracture.

The reduction in PTH level, although not significant, and its correlation with the degree of OB precursor maturation suggest that endogenous PTH is involved in the physiological control of fracture healing and in OB precursor maturation.

TGF $\beta$ 1 levels increased after fracture. Platelets have been shown to release this factor during the initial inflammatory phase of bone healing [31, 32], and it may thus be involved in initiating callus formation. Recent studies have suggested that it may act with IGF-1 released by bone implants in stimulating callus formation [33]. Here we demonstrate an increase in TGF $\beta$ 1 15 days after fracture in corroboration of its role in human fracture healing. We also found a strong inverse correlation between TGF $\beta$ 1 and OB precursors at baseline, whereas it is positively correlated to the degree of maturation of OB precursors (OC MIF). This positive correlation may account for the increased homing ability of circulating precursors [7]. The homing ability of circulating osteogenic cells during fracture healing was elegantly demonstrated by Kumagai et al. in a parabiotic mice model [12]. They showed that these cells are physiologically mobilized to contribute to callus formation and fracture repair. Enhanced homing ability may account for the absence of a universal increase in our patients

It has been shown (mainly in animal models) that the time needed for bone formation to bridge a fracture gap increases with age [34]. The reason for this slowing of fracture healing is not fully understood. Up-regulation of genes related to fracture healing has recently been shown in the callus of older animals. Meyer et al. [29, 30] reported up-regulation of TGF $\beta$  in the callus of older rats. This can be seen as an attempt by the tissue to accelerate the healing process. Our data confirm these findings as in older patients we observe a higher increase of TGF $\beta$ 1 and BAP after fracture; whereas age is not correlated with bone cell precursors. TGF $\beta$ 1 is directly correlated with the degree of OB precursors maturation even after correction for age. Its enhancement of OB precursors homing ability is presumably not age-dependent.

In conclusion, although the cohort observed is small, we suggest PTH as a possible player in the fracture healing process, and confirm the role of TGF $\beta$ 1 in the recruitment of osteogenic cells to the fracture site and its increase in function of age. On the other hand, we found no significant variation in the number of circulating bone cell precursors after fracture.

Further studies are needed to clarify the complex process of fracture healing and the role of osteogenic cell recruitment from the circulation.

**Acknowledgments:** This work was supported by a grant from the Fondazione Internazionale Ricerche Medicina Sperimentale (FIRMS) Compagnia San Paolo. P. D'Amelio was supported by a fellowship from the Regione Piemonte.

**Conflict of interest:** no disclosure.

## REFERENCES.

- [1] De Vries T.J., Everts V, Osteoclast Formation from Peripheral Blood of Patients with Bone-lytic Diseases (2009)
- [2] D'Amelio P., Grimaldi A., Di Bella S., Tamone C., Brianza S.Z., Ravazzoli M.G., Bernabei P., Cristofaro M.A., Pescarmona G.P., Isaia G., Risedronate reduces osteoclast precursors and cytokine production in postmenopausal osteoporotic women, *J Bone Miner Res* (2008) 23:373-9.
- [3] D'Amelio P., Grimaldi A., Cristofaro M.A., Ravazzoli M., Molinatti P.A., Pescarmona G.P., Isaia G.C., Alendronate reduces osteoclast precursors in osteoporosis, *Osteoporos Int* (2009)
- [4] Gossl M., Modder U.I., Atkinson E.J., Lerman A., Khosla S., Osteocalcin expression by circulating endothelial progenitor cells in patients with coronary atherosclerosis, *J Am Coll Cardiol* (2008) 52:1314-25.
- [5] Eghbali-Fatourehchi G.Z., Lamsam J., Fraser D., Nagel D., Riggs B.L., Khosla S., Circulating osteoblast-lineage cells in humans, *N Engl J Med* (2005) 352:1959-66.
- [6] Eghbali-Fatourehchi G.Z., Modder U.I., Charatcharoenwitthaya N., Sanyal A., Undale A.H., Clowes J.A., Tarara J.E., Khosla S., Characterization of circulating osteoblast lineage cells in humans, *Bone* (2007) 40:1370-7.
- [7] Pirro M., Leli C., Fabbriani G., Manfredelli M.R., Callarelli L., Bagaglia F., Scarponi A.M., Mannarino E., Association between circulating osteoprogenitor cell numbers and bone mineral density in postmenopausal osteoporosis, *Osteoporos Int* (2009)
- [8] Tsirodis E., Upadhyay N., Giannoudis P., Molecular aspects of fracture healing: which are the important molecules?, *Injury* (2007) 38 Suppl 1:S11-25.
- [9] Ferrara N., Davis-Smyth T., The biology of vascular endothelial growth factor, *Endocr Rev* (1997) 18:4-25.
- [10] Devine M.J., Mierisch C.M., Jang E., Anderson P.C., Balian G., Transplanted bone marrow cells localize to fracture callus in a mouse model, *J Orthop Res* (2002) 20:1232-9.
- [11] Shirley D., Marsh D., Jordan G., McQuaid S., Li G., Systemic recruitment of osteoblastic cells in fracture healing, *J Orthop Res* (2005) 23:1013-21.
- [12] Kumagai K., Vasanji A., Drazba J.A., Butler R.S., Muschler G.F., Circulating cells with osteogenic potential are physiologically mobilized into the fracture healing site in the parabiotic mice model, *J Orthop Res* (2008) 26:165-75.

- [13] Lieberman J.R., Daluiski A., Einhorn T.A., The role of growth factors in the repair of bone. Biology and clinical applications, *J Bone Joint Surg Am* (2002) 84-A:1032-44.
- [14] Sandberg M.M., Aro H.T., Vuorio E.I., Gene expression during bone repair, *Clin Orthop Relat Res* (1993) 292-312.
- [15] Pfeilschifter J., Oechsner M., Naumann A., Gronwald R.G., Minne H.W., Ziegler R., Stimulation of bone matrix apposition in vitro by local growth factors: a comparison between insulin-like growth factor I, platelet-derived growth factor, and transforming growth factor beta, *Endocrinology* (1990) 127:69-75.
- [16] Frenz D.A., Williams J.D., Van de Water T.R., Initiation of chondrogenesis in cultured periosteal mesenchyme. Synergistic action of transforming growth factor-beta and fibroblast growth factor, *Ann N Y Acad Sci* (1991) 630:256-8.
- [17] Joyce M.E., Roberts A.B., Sporn M.B., Bolander M.E., Transforming growth factor-beta and the initiation of chondrogenesis and osteogenesis in the rat femur, *J Cell Biol* (1990) 110:2195-207.
- [18] Cipriano C.A., Issack P.S., Shindle L., Werner C.M., Helfet D.L., Lane J.M., Recent Advances Toward the Clinical Application of PTH (1-34) in Fracture Healing, *HSS J* (2009) 5:149-53.
- [19] D'Amelio P., Grimaldi A., Pescarmona G.P., Tamone C., Roato I., Isaia G., Spontaneous osteoclast formation from peripheral blood mononuclear cells in postmenopausal osteoporosis, *Faseb J* (2005) 19:410-2.
- [20] D'Amelio P., Grimaldi A., Di Bella S., Brianza S.Z., Cristofaro M.A., Tamone C., Giribaldi G., Ulliers D., Pescarmona G.P., Isaia G., Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: a key mechanism in osteoporosis, *Bone* (2008) 43:92-100.
- [21] Ritchlin C.T., Haas-Smith S.A., Li P., Hicks D.G., Schwarz E.M., Mechanisms of TNF-alpha- and RANKL-mediated osteoclastogenesis and bone resorption in psoriatic arthritis, *J Clin Invest* (2003) 111:821-31.
- [22] Dalbeth N., Smith T., Nicolson B., Clark B., Callon K., Naot D., Haskard D.O., McQueen F.M., Reid I.R., Cornish J., Enhanced osteoclastogenesis in patients with tophaceous gout: urate crystals promote osteoclast development through interactions with stromal cells, *Arthritis Rheum* (2008) 58:1854-65.
- [23] Ramnaraine M., Pan W., Clohisy D.R., Osteoclasts direct bystander killing of cancer cells in vitro, *Bone* (2006) 38:4-12.
- [24] Massey H.M., Flanagan A.M., Human osteoclasts derive from CD14-positive monocytes, *Br J Haematol* (1999) 106:167-70.

- [25] Shalhoub V., Elliott G., Chiu L., Manoukian R., Kelley M., Hawkins N., Davy E., Shimamoto G., Beck J., Kaufman S.A., Van G., Scully S., Qi M., Grisanti M., Dunstan C., Boyle W.J., Lacey D.L., Characterization of osteoclast precursors in human blood, *Br J Haematol* (2000) 111:501-12.
- [26] Faust J., Lacey D.L., Hunt P., Burgess T.L., Scully S., Van G., Eli A., Qian Y., Shalhoub V., Osteoclast markers accumulate on cells developing from human peripheral blood mononuclear precursors, *J Cell Biochem* (1999) 72:67-80.
- [27] Matayoshi A., Brown C., DiPersio J.F., Haug J., Abu-Amer Y., Liapis H., Kuestner R., Pacifici R., Human blood-mobilized hematopoietic precursors differentiate into osteoclasts in the absence of stromal cells, *Proc Natl Acad Sci U S A* (1996) 93:10785-90.
- [28] Roato I., Grano M., Brunetti G., Colucci S., Mussa A., Bertetto O., Ferracini R., Mechanisms of spontaneous osteoclastogenesis in cancer with bone involvement, *Faseb J* (2005) 19:228-30.
- [29] Meyer M.H., Meyer R.A., Jr., Genes with greater up-regulation in the fracture callus of older rats with delayed healing, *J Orthop Res* (2007) 25:488-94.
- [30] Meyer R.A., Jr., Meyer M.H., Tenholder M., Wondracek S., Wasserman R., Garges P., Gene expression in older rats with delayed union of femoral fractures, *J Bone Joint Surg Am* (2003) 85-A:1243-54.
- [31] Bostrom M.P., Expression of bone morphogenetic proteins in fracture healing, *Clin Orthop Relat Res* (1998) S116-23.
- [32] Connor J.M., Evans D.A., Fibrodysplasia ossificans progressiva. The clinical features and natural history of 34 patients, *J Bone Joint Surg Br* (1982) 64:76-83.
- [33] Bernstein A., Mayr H.O., Hube R., Can bone healing in distraction osteogenesis be accelerated by local application of IGF-1 and TGF-beta1?, *J Biomed Mater Res B Appl Biomater* (2010) 92:215-25.
- [34] Meyer R.A., Jr., Tsahakis P.J., Martin D.F., Banks D.M., Harrow M.E., Kiebzak G.M., Age and ovariectomy impair both the normalization of mechanical properties and the accretion of mineral by the fracture callus in rats, *J Orthop Res* (2001) 19:428-35.

**TABLES.**

**Table 1.** Types of fracture

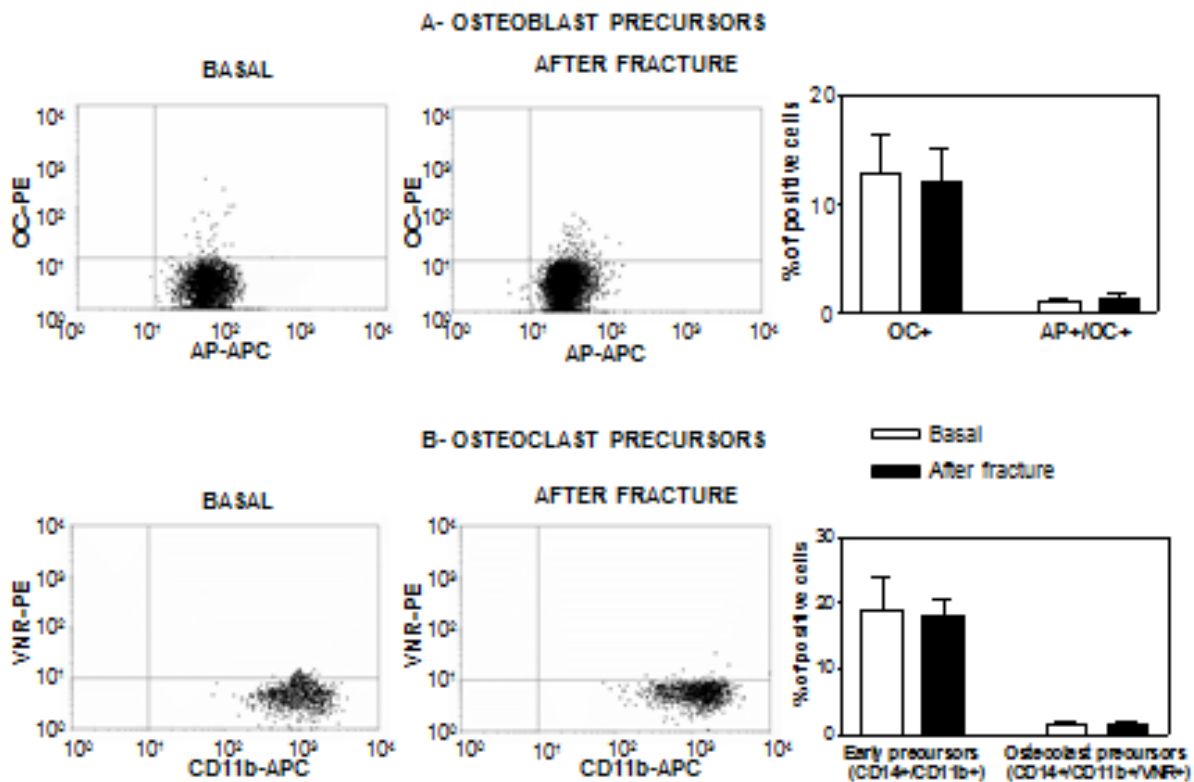
<b>Patient</b>	<b>Type of fracture</b>
FG	Lumbar vertebra (L1) and ileopubic left branch
AC	Right femur diaphysis
LG	Tibial and peroneal diaphysis
FF	Left radius
AN	Left radius
GR	Tibial diaphysis and heel
PP	Tibial diaphysis
PP1	Ileopubic branch left and right
DS	Left femur diaphysis

**FIGURE.**

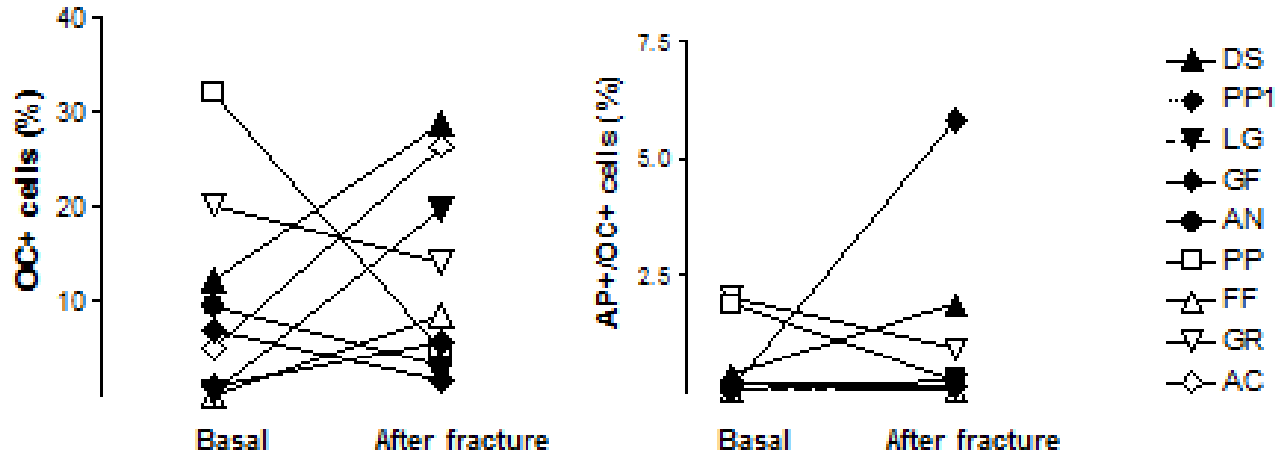
**Figure 1.** Bone cell precursors during fracture healing: FACS analysis of circulating osteoblast and osteoclast precursors from PBMCs of patients at baseline and 15 days after fracture.

**A.** Dot plots represent OC+/AP+/CD15- cells gated on monocytes (OB precursors). The graph represents the percentage of OC+ cells and AP+/OC+ cells at baseline and 15 days after fracture. The bars show the mean and SE for all patients, the differences are not significant according to the Wilcoxon-Mann-Whitney's test.

**B.** Dot plots represent VNR+ and CD11b+ cells gated on CD14+ cells (osteoclast precursors). The graph represents the percentage of CD14+/CD11b+/VNR- cells (early osteoclast precursors) and the percentage of CD14+/CD11b+/VNR+ cells (osteoclast precursors) at baseline and 15 days after fracture. The bars show the mean and SE for all patients, the differences are not significant according to the Wilcoxon-Mann-Whitney test.



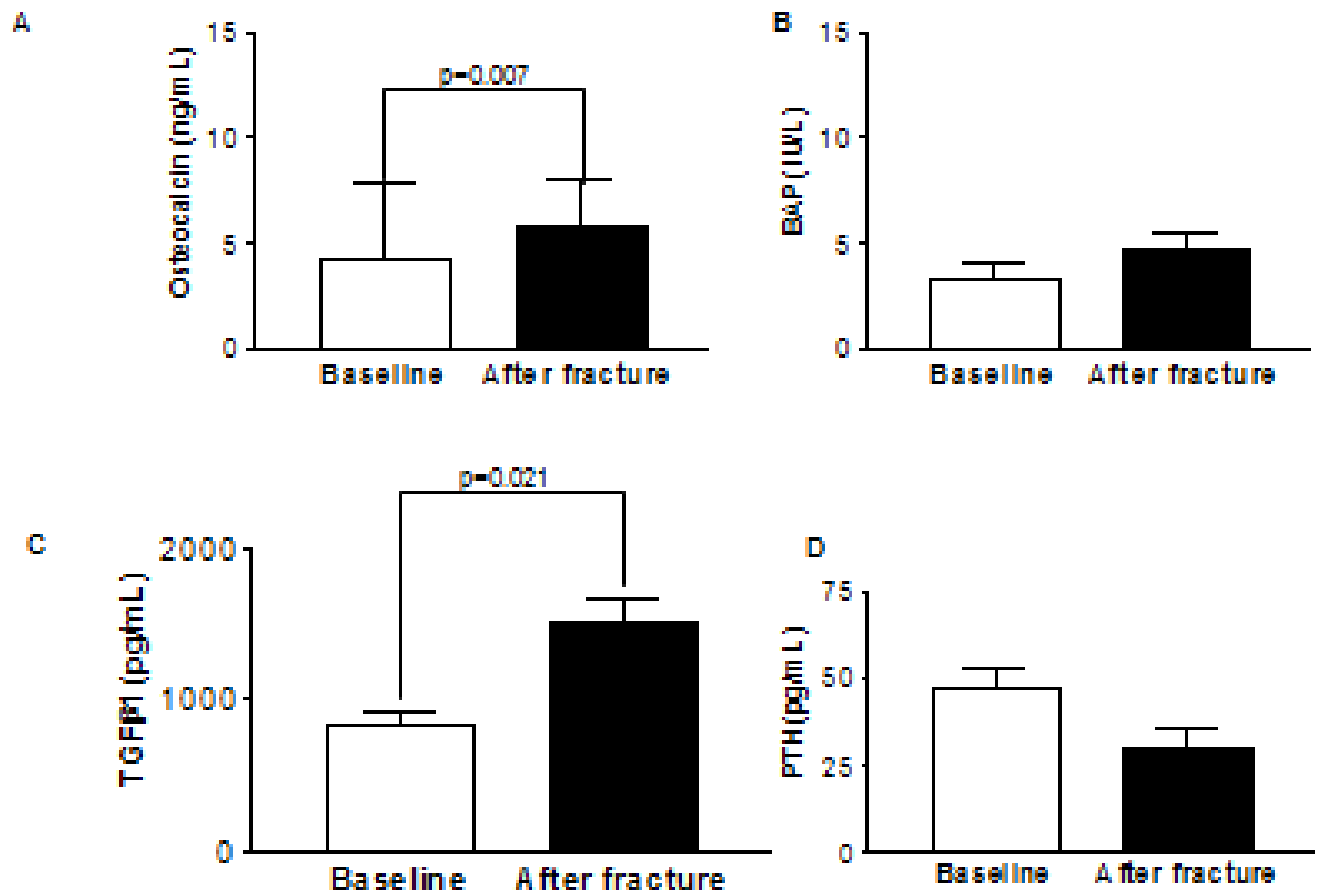
**Figure 2.** OB precursors at baseline and after fracture. The graphs show the percentage of OC+ cells (left) and of AP+/OC+ cells (right) in each patient at baseline and 15 days after fracture. Patients initials are indicated.



**Figure 3.** Effects of fracture healing on bone formation markers, TGFβ1 and PTH.

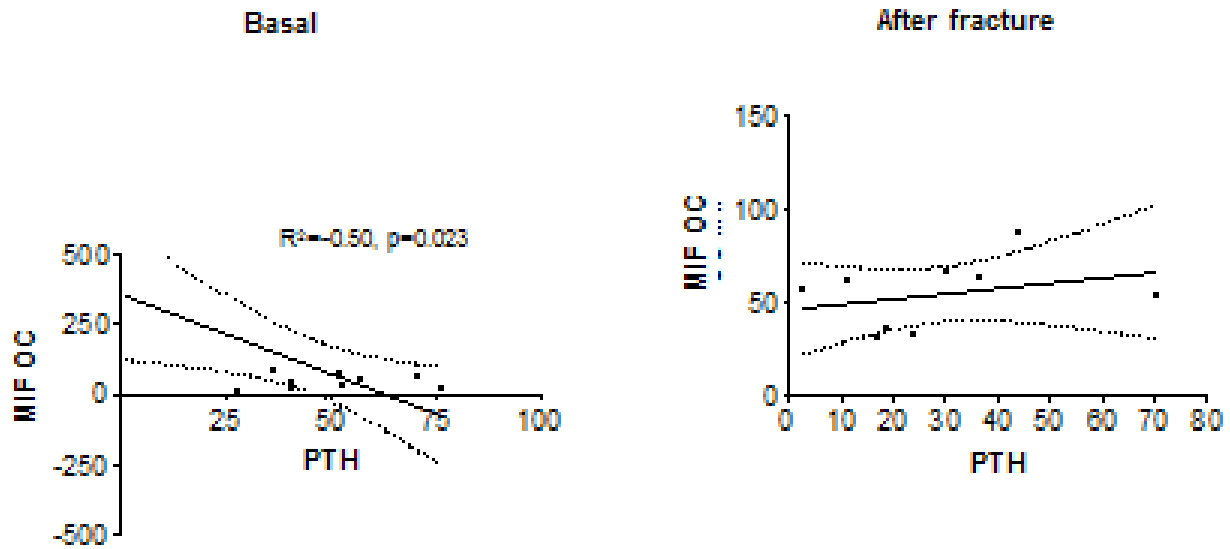
- B. Serum OC at baseline and 15 days after fracture
- C. Serum BAP at baseline and 15 days after fracture
- D. Serum TGFβ1 at baseline and 15 days after fracture
- E. Serum PTH at baseline and 15 days after fracture

The bars show the mean and SE for all patients. p significance values calculated with the Wilcoxon-Mann-Whitney test are indicated.



**Figure 4.** Correlations between PTH and degree of OB precursor maturation at baseline and after fracture.

Linear correlation between PTH and the mean fluorescence intensity (MIF) of OC at baseline (left) and 15 days after fracture (right). The dotted lines represent the confidence intervals, R and p values calculated by Spearman's test are indicated. The MIF indicates the degree of maturation towards osteoblasts of OB precursor cells.



**Figure 5.** Correlations between the percentage-fold changes of TGFβ1, BAP, PTH between baseline and 15 days after fracture and age.

A. Linear correlation between the percentage-fold change of TGFβ1 between baseline and 15 days after fracture and age

B. Linear correlation between the percentage-fold change of BAP between baseline and 15 days after fracture and age

C. Linear correlation between the percentage-fold change of PTH between baseline and 15 days after fracture of PTH and age

The dotted lines represent the confidence intervals; significant R and p values calculated by Spearman's test are indicated.

