

## Basic Study

## Evaluation of bone remodeling in regard to the age of scaphoid non-unions

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### Abstract

**AIM:** To analyse bone remodeling in regard to the age of scaphoid non-unions (SNU) with immunohistochemistry.

**METHODS:** Thirty-six patients with symptomatic SNU underwent surgery with resection of the pseudarthrosis. The resected material was evaluated histologically after staining with hematoxylin-eosin (HE), tartrate resistant acid phosphatase (TRAP), CD 68, osteocalcin (OC) and osteopontin (OP). Histological examination was performed in a blinded fashion.

**RESULTS:** The number of multinuclear osteoclasts in the TRAP-staining correlated with the age of the SNU and was significantly higher in younger SNU ( $P = 0.034$ ;  $r = 0.75$ ). A higher number of OP-immunoreactive osteoblasts significantly correlated with a higher number of OC-immunoreactive osteoblasts ( $P = 0.001$ ;  $r = 0.55$ ). Furthermore, a greater number of OP-immunoreactive osteoblasts correlated significantly with a higher number of OP-immunoreactive multinuclear osteoclasts ( $P = 0.008$ ;  $r = 0.43$ ). SNU older than 6 mo showed a significant decrease of the number of fibroblasts ( $P = 0.04$ ). Smoking and the age of the patients had no influence on bone remodeling in SNU.

**CONCLUSION:** Multinuclear osteoclasts showed a significant decrease in relation to the age of SNU. However, most of the immunohistochemical findings of bone remodeling do not correlate with the age of the SNU. This indicates a permanent imbalance of bone formation and resorption as indicated by a concurrent increase in both osteoblast and osteoclast numbers. A clear histological differentiation into phases of bone remodeling in SNU is not possible.

**Key words:** Bone remodeling; Histology; Immunohistological staining; Scaphoid non-union; Scaphoid; Wrist joint

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**Core tip:** The bone remodeling in regard to the age of scaphoid non-union is investigated with immunohistochemistry. Multinuclear osteoclasts showed a significant decrease in relation of the age of scaphoid non-union, but smoking and the age of the patients had no influence on bone remodeling. Most of the immunohistochemical findings of bone remodeling do not correlate with the age of the scaphoid non-unions, which indicates a permanent imbalance of bone formation and resorption.

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## INTRODUCTION

The scaphoid is the most commonly fractured carpal bone<sup>[1-3]</sup>. Scaphoid non-union (SNU) occurs in approximately 5% to 13% of treated scaphoid fractures and in an unknown number of unrecognized fractures<sup>[2,4-6]</sup>. The reasons for this high non-union rate are multifactorial including fracture location and vascularity, failure of recognizing the fracture and inadequate initial treatment<sup>[7,8]</sup>. Additionally, scaphoid fractures heal

by intramembranous ossification, which leaves the scaphoid without protective fracture callus against potentially disruptive forces. Progressive osteoarthritis, so called scaphoid non-union advanced collapse, inevitably develops in all cases with untreated SNU over time<sup>[9]</sup>.

Fracture healing consists of a regulatory circuit, which requires the proliferation and differentiation of osteoblasts and osteoclasts for bone regeneration and remodeling, together with formation of new blood vessels for bone vascularisation and a myriad of intercellular interactions and molecular communications to coordinate this complex process<sup>[10]</sup>. Osteoblasts produce organic components of the extracellular matrix, regulate the mineralisation of the osteoid and therefore are essential for bone formation<sup>[11,12]</sup>. Osteoclasts are responsible for bone resorption by removing mineralized matrix and breaking up the organic bone. Both osteoclasts and activated macrophages show a high expression of tartrate resistant acid phosphatase (TRAP) and glycoprotein CD 68. TRAP is synthesized as latent proenzyme and activated by proteolytic cleavage and reduction<sup>[13,14]</sup>. Osteocalcin (OC) is an extracellular matrix protein produced by osteoblasts, which constitutes 2% of the total protein content in bone. It is distributed in cement lines of both cortical and trabecular bone<sup>[15,16]</sup>. OC is thought to have a role in the early stages of bone healing and is a marker for bone formation<sup>[17,18]</sup>. Osteopontin (OP) is a non-collagenous extracellular matrix protein and is biosynthesized by osteoblasts, osteoclasts, osteocytes, activated fibroblasts, hypertrophic chondrocytes and cemented lines<sup>[16]</sup>. It is a multifunctional protein that is involved in several aspects of bone turnover and remodeling as well as fracture healing<sup>[16,19]</sup>.

A recent study found significant less bone remodeling in SNU older than a mean age of 45 mo<sup>[20]</sup>. However, conventional histological investigation is not sufficient to analyze bone remodeling, because staining of the tissue is unspecific. Immunohistochemistry (IHC) using specific markers of bone resorption and bone formation is helpful to shed further light on the process of bone remodeling in SNU. It is hypothesized that there would be differences in numbers of immunohistochemically stained cells that would correlate with the age of the SNU. This difference in staining may lead to the identification of more bone formation (increase in osteoblasts) or bone resorption (increase in number of osteoclasts) over time and may generate more information about the development of SNU. Therefore the aim of this study was to evaluate bone remodeling of SNU with immunohistochemical markers in regard to the age of the fracture.

## MATERIALS AND METHODS

### Ethics

The study was conducted in accordance with the Helsinki Declaration. The local ethics committee review

board approved the study (367/2007A).

### Patients

Thirty six male patients with a mean age of 26 (SD 12; range: 12-56) years at the time of injury were included in this study. Sixteen right and 20 left wrists were injured. The mean time between injury and surgery for non-union was 22 (SD 27; range: 4-144) mo. Six SNU were localised in the proximal third, 27 in the middle third, and one in the distal third of the scaphoid, respectively. In two patients, exact localisation of SNU was not defined. No additional surgery was performed during the follow-up period in 25 cases. However, one patient received a vascularised bone graft from the distal radius, one patient a four corner fusion, one patient a denervation of the wrist, and four patients another kind of wrist surgery in the postoperative follow-up. The data of the longer postoperative period in four patients were not available. Seventeen patients were non-smokers, 16 patients were smokers, but in three patients it was unclear, whether they are smokers or non-smokers.

Only patients, who stated a defined date of trauma having a symptomatic SNU, were included in this study. Exclusion criteria were unclear date of trauma, prior surgical treatment or associated adjacent injuries of the wrist as well as relevant underlying clinical diseases as diabetes mellitus or vascular disorders. Delayed fracture healing was defined between 4 to 6 mo. If no stable ossification was seen after 6 mo, the term non-union was used<sup>[21]</sup>.

### Histological examination

During surgery the SNU was resected completely, whereas resection sides showed healthy bone verified by macroscopic bleeding. Autologous cancellous bone was interposed in the former SNU gap and compression osteosynthesis using a Herbert screw was performed<sup>[22]</sup>. Specimens were immediately fixed in 4% neutral buffered (pH = 7.4) formaldehyde solution for 24 h at 4 °C, decalcified with diaminoethanetetraacetic acid and embedded in paraffin.

Sections of 2 µm were cut on a Leica rotation microtome (RM2055, Wetzlar, Germany) and mounted on silane-coated slides for conventional staining, enzyme- and IHC. HE-staining was performed in all specimens for morphological evaluation. Subsequently, the tissue sections were stained with TRAP, CD 68 (working dilution: 1:150, monoclonal, clone: KP-1, mouse anti-human, Dako, Glostrup, Denmark), OC (working dilution: 1:250, monoclonal, clone: OCG-3, mouse anti-human, Zytotec, Berlin, Germany) and OP (working dilution: 1:300, polyclonal, rabbit antisera, Chemicon, Temecula, Canada). Blocks and slides were stored at room temperature.

The mounted sections were dehydrated beginning with xylol in decreasing concentrations. Sections were then rehydrated with distilled water and pretreated according to the individual instructions from the suppliers of the used primary antibodies.

No special pretreatment was necessary for CD 68 and OC. For OP, specimens were treated with trypsin (pH = 6.0) for 30 min. After washing in phosphate-buffered saline solution (PBS, pH = 7.4), endogenous peroxidase activity was blocked in all sections with 1% hydrogen peroxide for 5 min. Nonspecific electrostatic protein charging was blocked with blocking reagent (Dako, Glostrup, Denmark) for 10 min at room temperature. Sections were incubated with respective normal sera (Linaris, Wertheim, Germany) for an hour at room temperature and then incubated overnight at 37 °C with primary antibodies. Biotinylated secondary antibodies were added for 30 min at 37 °C, followed by an avidin-biotin-enzyme complex for 30 min at 37 °C (Vectastain ABC-HRP kit, Linaris, PK-4000, Wertheim-Bettingen, Germany) at room temperature. The peroxidase activity was visualized with 3'-3'-diaminobenzidine. Then counterstaining with hematoxylin was performed. Sections were washed thoroughly three times in PBS for 5 min after each step. Finally, sections were dehydrated and covered with Entellan (Merck, Darmstadt, Germany). Control procedures, *i.e.*, identical staining without adding primary antibodies, were performed in parallel. Then counterstaining with hematoxylin was performed.

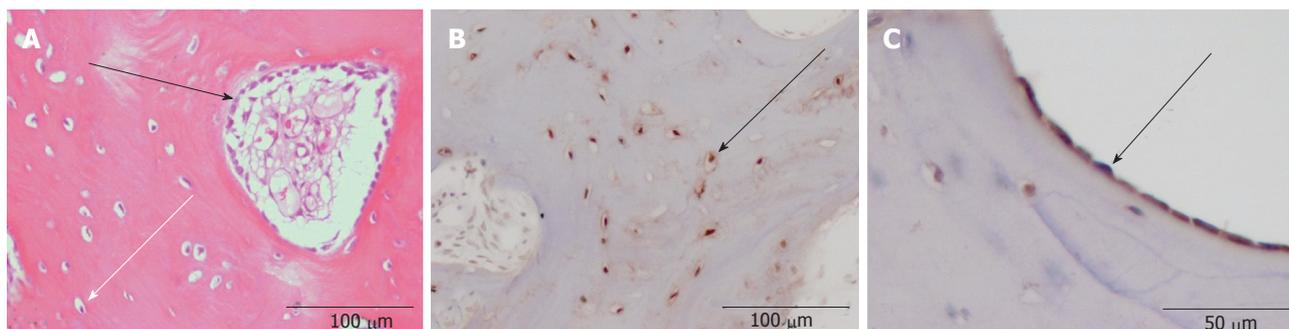
Histopathological examination of the stained tissue sections was performed using an Olympus BHS light microscope in the transmitted mode at final magnifications of 40 ×, 100 ×, 200 × and 400 ×. One section in each staining per subject was analysed. Total cell counts were counted at an original magnification of 100 × in 10 subsequent adjacent visual fields, representing the whole width of the non-union. Only fibroblasts were counted in 5 subsequent visual fields, because the volume of fibroblast tissue was not big enough for 10 visual fields in most cases. All specimens were blinded for cell counts.

Histopathological analysis was centered on osteopathological criteria including determination of chondrocytes and extracellular matrix (ECM) at the non-union gap, osteoblasts, osteoclasts, osteocytes, osteoid and cement lines of the underlying bone, mesenchymal cells and ECM in resorptive bone cysts as well as cysts containing fibrous or fibrocartilage tissue and typical hyaline cartilage in the OC and OP staining<sup>[23]</sup>.

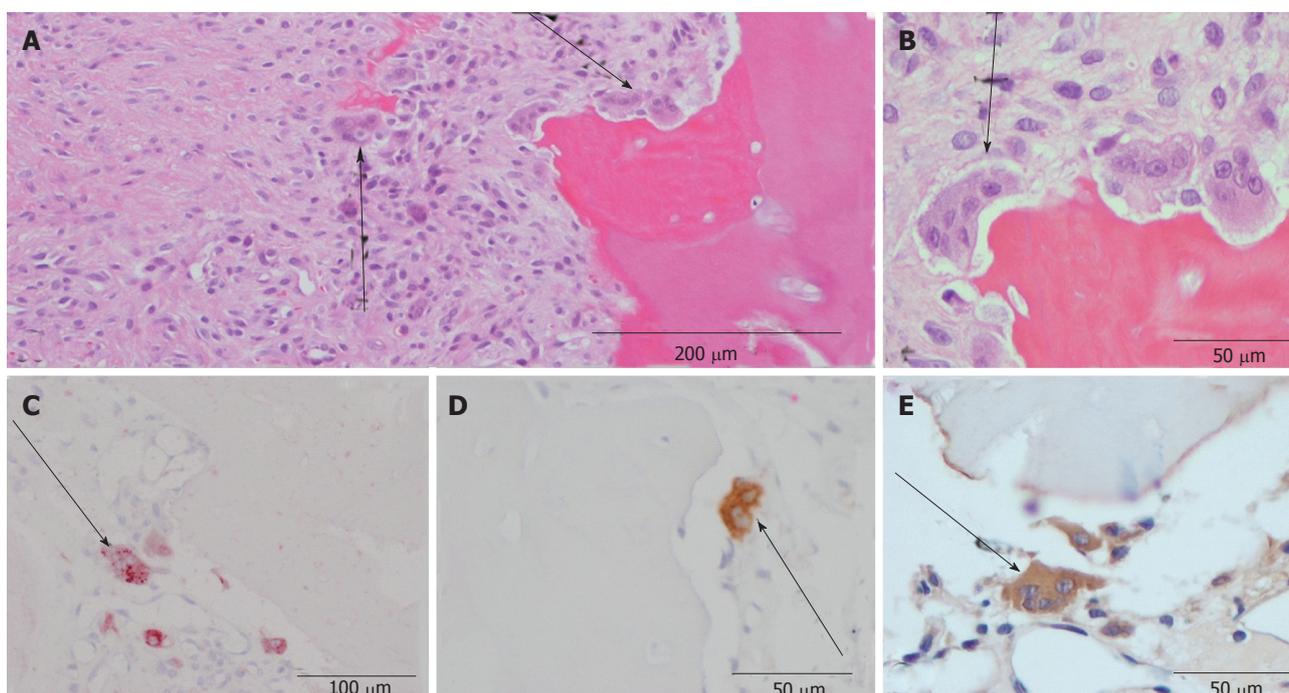
### Morphological analysis and cell counting

Morphological analysis was first performed with the HE-staining. With the help of the following criteria the different cell types were identified: Osteoblasts, which are mononuclear cells, were counted if they lined up the external surface of bone trabeculae and the surface of Haversian canals (Figure 1)<sup>[23,24]</sup>. Osteocytes were counted if they were embedded into the mineralized bone matrix (Figure 1). Cells with two to fifteen nuclei in small resorptive excavations (Howship's lacunae) on the bone surface were counted as multinuclear osteoclasts (Figure 2).

Mononuclear osteoclast precursors could only be



**Figure 1 Morphological analysis.** A 7-mo-old SNU is shown (A-C). A: HE-staining shows osteoblasts (black arrow) and osteocytes (white arrow) in the underlying sclerosed bone; B: OC-staining shows osteocytes (arrow) in detail; C: OP-staining shows the osteoblasts (arrow) lining a Haversian vessel in detail. Original magnification 200 × (A, B), 400 × (C). SNU: Scaphoid non-unions; OP: Osteopontin; OC: Osteocalcin.



**Figure 2 Osteoclasts.** Examples for multinuclear osteoclasts as seen in the HE-staining (A and B), the TRAP (C), CD 68 (D) and OP (E) staining. (A-D) show osteoclasts (arrows) in a 10-mo-old SNU, whereas (E) shows an osteoclast in a 7-mo-old SNU. Original magnification 100 × (A), 200 × (C), 400 × (B, D, E). SNU: Scaphoid non-unions; OP: Osteopontin; TRAP: Tartrate resistant acid phosphatase.

identified in the TRAP and CD 68 staining and were counted as mononuclear positive-stained cells in these two stainings. Fibroblasts were counted as cells located in the non-union gap in the HE-staining (Figure 3).

Multinuclear osteoclasts were counted in the TRAP staining, with CD 68 and OP-IHC. Osteoblasts and osteocytes were counted with OP- and OC-IHC, respectively.

### Data analysis

Statistical analysis was performed with a two sided *t* test in order to analyse the occurrence of OC- and OP-positive cells in the different parts of the SNU with a level of significance of  $P \leq 0.05$ .

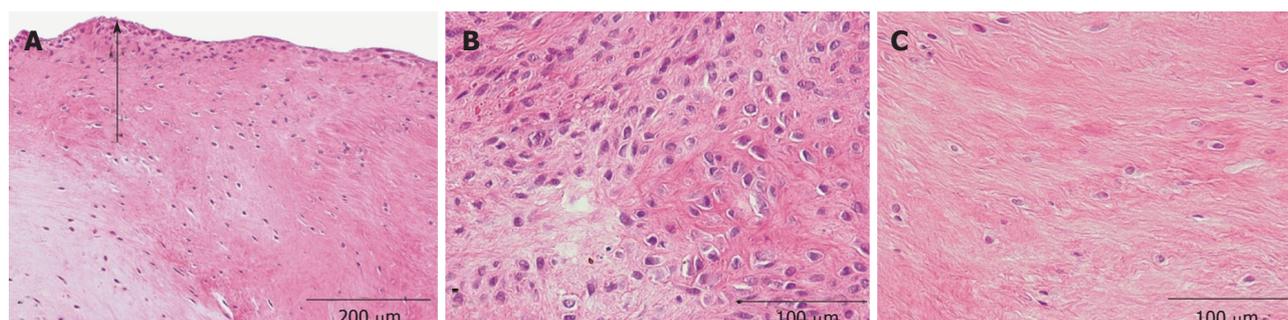
The two-sided Pearson correlation analysis was used to investigate the linear relationship with regard to age of

SNU and patients, and counted cell numbers. Correlation analysis was performed with Spearman's rho coefficient with a significance level of  $P \leq 0.05$ . The influence of smoking has been investigated with the Kruskal-Wallis test followed by the Mann-Whitney test with a level of significance of  $P \leq 0.05$ . Statistical analysis was performed with the computer program SPSS (Version 11.5, Chicago, United States).

## RESULTS

### Immunohistochemical findings

Table 1 gives an overview over the markers that could reliably and reproducibly be detected. Negative procedures without antibodies showed no staining. OP was immunolocalized within chondrocytes and ECM



**Figure 3 Non-union tissue in the hematoxylin-eosin staining.** A: Non-union tissue with fibrocartilage covered by a layer of synovium-like lining cells (arrow) of a 60-mo-old SNU; B: Fibrous tissue rich in fibroblasts of a 10-mo-old SNU. Note the contrast in cell number as compared to the 60-mo-old SNU in A; C: Fibrous tissue with few fibroblasts, same patient as in A. Original magnification 100 × (A), 200 × (B, C). SNU: Scaphoid non-unions.

**Table 1 Summary of the histological features with the range of time of their appearance**

Histological feature		Immunohistochemical findings													
		Osteocalcin						OP							
		Yes (n)	Range of time (mo)		No (n)	Range of time (mo)		Not assessable (n)	Yes (n)	Range of time (mo)		No (n)	Range of time (mo)		Not assessable (n)
Non-union	Chondrocytes	1	12	12	35	4	144	-	25	4	144	11	6	60	-
	ECM	0	-	-	36	4	144	-	34	4	144	2	15	24	-
Underlying bone	Osteoblasts	31	6	144	4	4	22	1	34	4	144	2	9	15	-
	Osteoclasts	2	8	29	33	4	144	1	33	4	85	2	9	15	1
	Osteocytes	32	6	144	4	4	15	-	34	4	144	2	9	15	-
	Osteoid	13	7	28	23	4	144	-	32 <sup>1</sup>	4	85	4 <sup>1</sup>	14	144	-
	Cement lines	33	4	144	3	7	15	-	8	7	144	28	4	85	-
Resorptive bone cysts	Mesenchymal Cells	-	-	-	15	4	60	21	11	4	24	5	9	60	20
	ECM	-	-	-	15	4	60	21	15	4	60	1	15	15	20
Fibrous bone cysts	Mesenchymal cells	1	15	15	12	7	54	23	10	7	54	7	4	28	19
	ECM	1	15	15	12	7	54	23	12	7	54	5	4	24	19
Hyaline cartilage	Typical	33	4	144	2	9	12	1	34	4	144	1	15	15	1

The underlying bone's osteoid showed significantly more often OP-immunoreactivity in younger ( $18.5 \pm 17.9$  mo) than in older SNU ( $50.5 \pm 62.7$  mo) ( $P = 0.02$ ). ECM: Extracellular matrix; OP: Osteopontin; SNU: Scaphoid non-unions.

of the non-union, osteoclasts, osteoblasts, osteoid and osteocytes of the underlying bone as well as the hyaline cartilage. Cement lines of newly formed lamellar bone only stained positively for OP in 8 out of 36 cases (Table 1). OC showed immunoreactivity in cement lines, osteocytes, osteoblasts and hyaline cartilage (Table 1). Resorptive and fibrous bone cysts showed immunoreactivity for OP in most cases but not for OC (Table 1). Enzyme-histochemical staining against TRAP specifically stained osteoclasts and mononuclear precursors indicating bone resorption during the remodeling process (Figure 2C). The macrophage marker CD 68 was detected in mononuclear and multinuclear macrophages or osteoclasts (Figure 2D). Mononuclear macrophages/osteoclast precursors and multinuclear osteoclasts stained positively for CD 68 in 32 out of 36 cases, whereas in only 8 out of 36 cases osteoclasts were stained positive for TRAP.

Osteoid showed immunoreactivity for OP in 32 younger SNU ( $18.5$  SD  $17.9$  mo) with a range of age

between 4 to 85 mo, whereas there was no immunoreactivity for OP in 4 older SNUs ( $50.5$  SD  $62.7$  mo) with a range of age between 14 to 144 mo. The difference between the two groups was statistical significant ( $P = 0.02$ ; Table 1).

**Cell counting and correlation analysis**

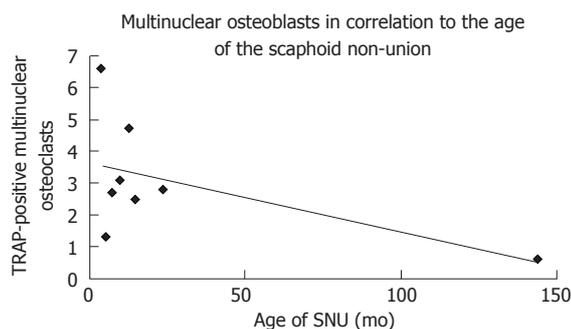
Single results of the cell counting are presented in Table 2. The number of multinuclear osteoclasts in the TRAP-staining correlated with the age of the SNU and was significantly higher in younger SNU ( $P = 0.034$ ;  $r = 0.75$ ; Figure 4). All other correlations in regard to the age of the SNU showed no significant results.

A higher number of OP-immunoreactive osteoblasts significantly correlated with a higher number of OC-immunoreactive osteoblasts ( $P = 0.001$ ;  $r = 0.55$ ; Figure 5). Furthermore, a greater number of OP-immunoreactive osteoblasts correlated significantly with a higher number of OP-immunoreactive multinuclear osteoclasts ( $P = 0.008$ ;  $r = 0.43$ ; Figure 6).

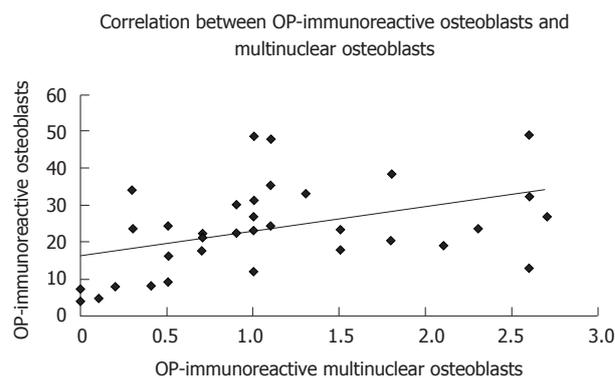
**Table 2** Results of the cell counting are shown as mean with standard deviation

Single results of the cell counting					
Histological feature	HE	TRAP ( <i>n</i> = 8)	CD 68 ( <i>n</i> = 32)	OP ( <i>n</i> = 36)	OC ( <i>n</i> = 36)
Osteoblasts	N/A	N/A	N/A	23.4 ± 12	25.6 ± 19.5
Osteoclasts					
Uninuclear	N/A	9 ± 6.5	4.6 ± 5.1	N/A	N/A
Multinuclear	N/A	2.3 ± 0.8	1.8 ± 1.2	1.1 ± 0.8	N/A
Osteocytes	N/A	N/A	N/A	38.2 ± 11.2	50.8 ± 16.4
Fibroblasts	285 ± 181	N/A	N/A	N/A	N/A

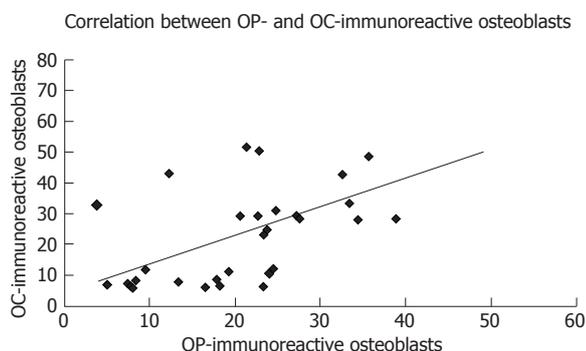
TRAP: Tartrate resistant acid phosphatase; CD 68: Cluster of differentiation 68; OC: Osteocalcin; OP: Osteopontin; N/A: Not analysed.



**Figure 4** Multinuclear osteoclasts in correlation to the age of the scaphoid non-unions. The number of multinuclear osteoclasts in the TRAP-staining was significantly higher in younger SNU ( $P = 0.034$ ;  $r = 0.75$ ). SNU: Scaphoid non-unions; TRAP: Tartrate resistant acid phosphatase.



**Figure 6** Correlation between osteopontin-immunoreactive osteoblasts and multinuclear osteoclasts. A greater number of OP-immunoreactive osteoblasts correlated significantly with a greater number of OP-immunoreactive multinuclear osteoclasts ( $P = 0.008$ ;  $r = 0.43$ ). OP: Osteopontin.



**Figure 5** Correlation between osteoblasts in the osteopontin- and osteocalcin- immunohistochemistry. A greater number of OC-immunoreactive osteoblasts correlated significantly with a greater number of OP-immunoreactive osteoblasts ( $P = 0.001$ ;  $r = 0.55$ ). OC: Osteocalcin; OP: Osteopontin.

A mean of 285 (SD 181) fibroblasts were counted in the 36 investigated SNU in the HE staining. A mean of 457 (SD 175) fibroblasts were counted in SNU ( $n = 4$ ) up to 6 mo old. In contrast, a mean of 264 (SD 173) fibroblasts were measured in SNU ( $n = 32$ ) older than 6 mo. This was a significant decrease of fibroblasts in SNU, which are older than 6 mo ( $P = 0.04$ ). However, no significant correlations have been found between the age of the patients and all investigated cell types. Furthermore, no significant differences have been observed between smokers and non-smokers for all investigated cell types.

## DISCUSSION

A recent study has shown that significant less bone remodeling takes place in older SNU with a mean age of 45 mo compared to a mean age of 18 mo<sup>[20]</sup>. However, these results were based on conventional HE-staining. Several bone-specific extracellular matrix proteins may be used to assess bone remodeling<sup>[16]</sup>. OC is reportedly the most specific noncollagenous bone matrix protein, being expressed by osteoblasts and osteocytes<sup>[25]</sup>. In the present study, we demonstrate specific staining of osteoblasts, osteocytes, cement lines, hyaline cartilage and in some cases osteoid ( $n = 13$ ). OP is reportedly expressed by osteocytes, osteoblasts, and their precursors, osteoclasts, hypertrophic chondrocytes, and cement lines<sup>[15,16]</sup>. We have seen specific staining of osteoblasts, osteoclasts, osteocytes, osteoid, chondrocytes, ECM of the non-union gap, and hyaline cartilage. OP interacts with osteoclasts, implicating it as a potentially important marker of bone resorption<sup>[26]</sup>.

A greater number of OC-immunoreactive osteoblasts correlated significantly with a greater number of OP-immunoreactive osteoblasts. However, correlation analysis between the two markers showed no time-dependent significant differences. Furthermore, a higher number of OP-immunoreactive osteoblasts correlated significantly with a higher number of OP-immunoreactive multinuclear osteoclasts, indicating a

higher bone remodeling in younger SNU. These findings confirm the theory that bone remodeling is a balance between bone formation and bone resorption in which osteoblasts exhibit two opposite phenotypes. There is the osteogenic phenotype, which secretes bone matrix at the bone resorption site, and the osteoclastogenic phenotype, which supports osteoclast differentiation in the old bone area<sup>[11]</sup>. The close interplay between osteoblasts and osteoclasts during bone repair is well established<sup>[10-12,17,23,24]</sup>.

A recent study has shown that cell viability and mineralization-positive colony forming units were significantly reduced in osteoblasts retrieved from non-union sites. This study identified a set of significantly down-regulated factors in those "non-union osteoblasts" that are involved in the regulation of osteoblast proliferation and differentiation<sup>[27]</sup>. This indicates that activity of osteoblasts in non-unions is altered, which could explain the lack of time-dependent changes in the OC- and OP-staining. However, another study could demonstrate, that OC-positive osteoblasts, which were taken from SNU, possessed osteogenic capability and could be stimulated by recombinant human bone morphogenetic protein-2 *in vitro*, resulting in significant increase in osteoblast differentiation and bone production<sup>[28]</sup>.

The number of osteoclasts decreased significantly in older SNU, which could be shown in the TRAP-staining, but not in the CD 68 IHC. This could be explained by the fact that CD 68 is not a specific osteoclast marker but rather a marker for several cells of the monocyte/macrophage lineage.

It is known, that nicotine has a dose dependent negative effect on bone healing, resulting in ischemia, diminished osteoblast function and decreased expression of bone morphogenetic protein<sup>[29,30]</sup>. However, in this study nicotine abuse had no measurable influence on bone remodeling in SNU.

The high account of fibroblasts reflects a cell rich fibrous tissue in the non-union gap. We have seen a significant decrease of the count of fibroblasts in SNU older than 6 mo. Our explanation is that the instability in the non-union gap induces or provokes an activation of fibroblasts. For that reason, further research on this topic could be the investigation of proliferation with specific immunohistochemical markers, *e.g.*, Ki 67.

A greater number of OP-immunoreactive osteoblasts significantly correlated with a greater number of OC-immunoreactive osteoblasts and OP-immunoreactive multinuclear osteoclasts. Multinuclear osteoclasts show a significant decrease in older SNU. Fibroblasts showed a significant decrease in SNU, which are older than 6 mo. These results indicate a decreased bone remodeling in older SNU. On the other hand, permanent remodeling indicates mechanical instability and imbalance. Therefore most of the immunohistochemical markers of bone remodeling do not correlate with the age of the SNU. Smoking had no influence on bone remodelling in SNU.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

The scaphoid is the most commonly fractured carpal bone, whereas non-union occurs in approximately 5% to 13% of treated scaphoid fractures.

### Research frontiers

Conventional histological stainings are insufficient to analyze bone remodeling, because staining of the tissue is unspecific.

### Innovations and breakthroughs

Immunohistochemistry using specific markers of bone resorption and bone formation is helpful to shed further light on the process of bone remodeling in scaphoid non-union.

### Applications

Multinuclear osteoclasts, as a marker for bone resorption, showed a significant decrease in relation of the age of scaphoid non-union, but smoking and the age of the patients had no influence on bone remodeling. Most of the immunohistochemical findings of bone remodeling do not correlate with the age of the scaphoid non-unions (SNU), which indicates a permanent imbalance of bone formation and resorption.

### Peer-review

This is a study on the bone remodeling in regard to the age of SNU with immunohistochemistry. The rationale for the study is appropriate and it is an interesting paper with a valuable contribution.

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