Different cytokine expression profiles in metaphyseal and diaphyseal fracture healing may provide new insights in the field of bone regeneration

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ABSTRACT:

Introduction Fractures are traumatic injuries that mainly occur in bone metaphysis, however most studies of bone healing have focused on diaphyseal bone. This is important because the healing process of trabecular metaphyseal bone has different healing characteristics from the diaphyseal area. Inflammation is thought to play an important, but different role in these two bone fracture types: diaphyseal fractures heal slowly through the formation of callus tissue, and metaphyseal trabecular bone heals faster, with no, or limited callus formation. As cytokines are key modulators of inflammation, the aim of the present study was to define the cytokine profiles at the core of these two conditions with possible implications for the bone healing process. **Materials and Methods** This study included sixteen patients with long bone metaphyseal and diaphyseal fractures and a healthy control group. Blood samples were taken at two timepoints: *i)* between the 1st (the day of the fracture) and the 6th day after fracture occurrence; *ii)* between the 7th and the 21st day after fracture occurrence. Fractures were treated either conservatively or surgically, depending on specific clinical indications. All participants with diaphyseal fractures were treated surgically. The control group provided blood samples on one occasion. The obtained plasma samples were pooled into 5 different experimental groups and analysed using commercial cytokine arrays. **Results** Marked differences in cytokine expression profiles were found between the fracture groups and the control group. The diaphyseal group had an "activated" pro-inflammatory cytokine profile with markedly higher levels of cytokines at both timepoints compared to the metaphyseal group, which in contrast had a "silenced" cytokine expression profile. Single cytokine analysis revealed that in both metaphyseal and diaphyseal fracture groups MCP-1 and RANTES showed the most prominent fold change at both timepoints. IL-6 and TNF-α also show similarly elevated levels in both timepoints in the diaphyseal fracture group, whereas this is not observed in the metaphyseal group. Furthermore, IL-3 expression was also elevated in the diaphyseal group, but only in the first timepoint. **Conclusion** This pilot study indicated chemokines which might be potential crucial drivers of bone healing, as well as painted distinct cytokine plasma profiles evident in metaphyseal and diaphyseal healing.

KEYWORDS: bone fractures, cytokines, fracture healing, bone regeneration

SAŽETAK:

RAZLIKE U EKSPRESIJI CITOKINA U CIJELJENJU PRIJELOMA KOŠTANE METAFIZE I DIJAFIZE PRUŽAJU MOGUĆE NOVE SPOZNAJE U PODRUČJU KOŠTANE REGENERACIJE

Uvod Koštani prijelomi su traumatske ozljede koje se većinom događaju u području koštane metafize. Međutim, većina istraživanja se usmjerava na dijafiznu kost. Važno je naglasiti da proces cijeljenja trabekularne kosti metafize ima različite karakteristike od dijafizne kosti. Pretpostavlja se da upala ima važnu, ali i različitu ulogu u ova dva tipa prijeloma: dijafizni prijelomi cijele sporije formacijom kalusnog tkiva, dok metafizni prijelomi cijele brže, s ograničenim stvaranjem kalusa ili bez njega. S obzirom na to da su citokini ključni modulatori upale, cilj ovog istraživanja je bio definirati profil citokina koji su u središtu ova dva procesa, s mogućom implikacijom na proces koštanog cijeljenja.

Materijali i metode U ovo istraživanje uključeno je šesnaest pacijenata s prijelomima duge kosti u području metafize ili dijafize te kontrolna grupa zdravih ispitanika. Uzorci krvi uzeti u dvije vremenske točke: *i*) između 1. (na dan prijeloma) i 6. dana poslije prijeloma; *ii*) između 7. i 21. dana poslije prijeloma. Prijelomi su liječeni konzervativno ili kirurški, ovisno o specifičnim kliničkim indikacijama; svi sudionici s dijafiznim prijelomima liječeni su kirurški. Uzorci krvi kontrolne skupine uzeti su u jednoj vremenskoj točki. Uzorci plazme podijeljeni su u 5 različitih eksperimentalnih skupina i analizirani koristeći komercijalno dostupne citokinske testove. **Rezultati** Pronađene su značajne razlike u profilu citokinske ekspresije skupina s prijelomima i kontrolne skupine. Skupina s dijafiznim prijelomima pokazala je "aktivirani" pro-upalni citokinski profil s naznačeno višim razinama citokina u obje vremenske točke, u usporedbi sa skupinom s metafiznim prijelomima, koja je pokazala "utišani" ekspresijski profil citokina. Analiza pojedinačnih citokina pokazala je da su MCP-1 i RANTES imali najizraženije povišen faktor promjene (eng. *fold change*) u obje skupine s koštanim prijelomima, i to u obje vremenske točke. IL-6 i TNF-α su pokazali sličan obrazac u obje vremenske točke u dijafiznoj skupini, dok u metafiznoj skupini to nije uočeno. Ekspresija IL-3 je također bila povišena u dijafiznoj skupini, ali samo u prvoj vremenskoj točki. **Zaključak** Ova pilot studija ukazala je na citokine koji bi potencijalno mogli biti važni čimbenici u koštanom cijeljenju uz prikaz različitih profila citokina plazme u metafiznom i dijafiznom koštanom cijeljenju.

KLJUČNE RIJEČI: koštani prijelomi, citokini, cijeljenje koštanih prijeloma, koštana regeneracija

INTRODUCTION

The bone is a highly specialized organ, characterized by hardness, rigidity, and power of repair and regeneration. It acts as a mineral reservoir for calcium homeostasis, acid-base buffer and a reservoir of growth factors and cytokines. Long bones are divided into epiphyses, which are placed at each bone end; metaphyses (rich with cancellous bone) that connect epiphyses with the cortical-rich central part of the bone – the diaphysis (1–3). Fractures are frequent traumatic injuries that most commonly occur in metaphyseal areas of long bones in humans and show different healing characteristics in comparison to diaphyseal fractures (3–5). The bone healing process is commonly didactically divided into four stages: It starts with *i)* hematoma formation and inflammation; followed by *ii)* soft (cartilagineous) callus formation, *iii)* bony callus formation and, finally, *iv)* bone remodeling (3,6). It is important to note that these classical four stages of bone healing pertain to diaphyseal bone healing on the periosteal side (7). Contrastingly, stable metaphyseal fractures are repaired by direct bone formation within the bone marrow; additionally, cartilaginous and bony callus formation are not observed on the periosteum side (3,5,8). In general, inflammation is thought to

play a crucial role in the initiation of fracture healing. Callus formation and resolution is coordinated by a complex and highly orchestrated network of molecular and cellular factors. Diaphyseal fractures mainly heal via the formation of callus tissue; whereas in metaphyseal spongy bone, limited or no callus is formed, and healing occurs by direct bone formation within the injured marrow compartment, which at the same time serves as a source of mesenchymal cells that are vital for bone regeneration (9). Furthermore, bone healing is also modulated by the intercellular communication between immune and mesenchymal stem cells (MSCs) (10). MSCs residing close to the bone surface show strong osteogenic potential in comparison to cells located in the central region of the bone marrow (11). Periosteum is the major source of cells with significant osteogenic and chondrogenic potential (12).

In diaphyseal fractures, one of the crucial events is the recruitment of competent cells from surrounding tissues or circulation, in which inflammation might play a key role (13). The inflammatory phase of fracture healing includes activation of innate immunity mechanisms and secretion of proinflamma-

tory cytokines, such as tumor necrosis factor alpha (TNF-α) and several interleukins (IL-1, IL-6, IL-11, IL-23), which are described as essential signals (14). The secreted cytokines attract immune cells involved in fracture healing, such as macrophages, monocytes and lymphocytes to the injury site, which in turn remove necrotic tissue and secrete growth factors, which further stimulates fracture healing (15). The importance of immunity in fracture healing has been demonstrated even in the hematoma phase of the fracture, as upregulation of regulatory T helper cells and anti-inflammatory cytokines (IL-10) coincided with an upregulation of angiogenic factors, which are essential for revascularisation (15). Pro-inflammatory cytokines are also highly expressed in later phases of fracture healing, including the remodeling phase (10). However, timely termination of inflammatory processes is of importance in normal bone healing, as prolonged pro-inflammatory signaling occurs in delayed bone-healing models (16). The importance of precise spatial and temporal regulation of inflammatory processes is strengthened by the fact that the administration of immunosuppressant dexamethasone, has strikingly different effects on healing in metaphyseal and diaphyseal fractures (17,18). Moreover, this also implies that there is functional heterogeneity in response to bone injury at the site of bone marrow mesenchymal cells and the periosteum; the metaphyseal region is more conducive to the recruitment of MSCs to the injury site (3). Considering these distinct patterns of bone healing, a cytokine expression profile with increased pro-inflammatory markers would be essential for cortical (diaphyseal), but possibly not for metaphyseal fracture healing.

Taking these observations in account; wishing to explore the systemic differences in cytokine plasma profiles between patients that suffered from either metaphyseal or diaphyseal long bone fractures, we conducted a small prospective observational study in which we analysed the expression of 23 different cytokines of patients' plasma at two distinct time points. This was done in order to further understand the role cytokines and immune mechanisms have in fracture healing, to establish differences in cytokine profiles among distinct types of fractures and their respective phases, as well as to potentially provide insights which can be translated into research uncovering modes of fracture healing optimisation.

PARTICIPANTS AND METHODS
Participants

This study included sixteen patients with long bone (N=10 with metaphyseal, N=6 with diaphyseal) fractures and a healthy control group (N=10), study outline is depicted in Figure 1. Subjects with bone fractures were recruited between January 1st and March 31st 2021 at the Clinic for Traumatology, Sisters of Charity University Hospital Centre, Zagreb. All participants included in the study have provided written consent for participation. The study was approved by the Ethics Committee of the Sisters of Charity University Hospital Centre. In order to be included in the study, all participants had to meet the inclusion criteria and none of the exclusion criteria (Table 1). Solely patients that suffered from long bone fractures (i.e., fractures of the metaphyseal or diaphyseal part of the radius, ulna, humerus, femur, tibia or fibula) were included. These types of fractures were selected because of their presumed similarity to animal models of fractures used in existing studies (19). The control group which consisted of ten (N=10) healthy volunteers who provided blood samples on one occasion. All healthy volunteers were female, aged 22-65. To be included in the study as healthy controls, participants had to be free of any active infections, malignant or other life-endangering disease. Samples were taken at two timepoints: i) the first timepoint between the 1st (the day of the fracture) and the 6th day after fracture occurrence; ii) the second timepoint was set between the 7th and the 21st day after fracture occurrence. Fractures were treated either conservatively or surgically, depending on specific clinical indications. All participants with diaphyseal fractures were treated surgically. Additional data about the participants can be found in Supplementary Table 1.
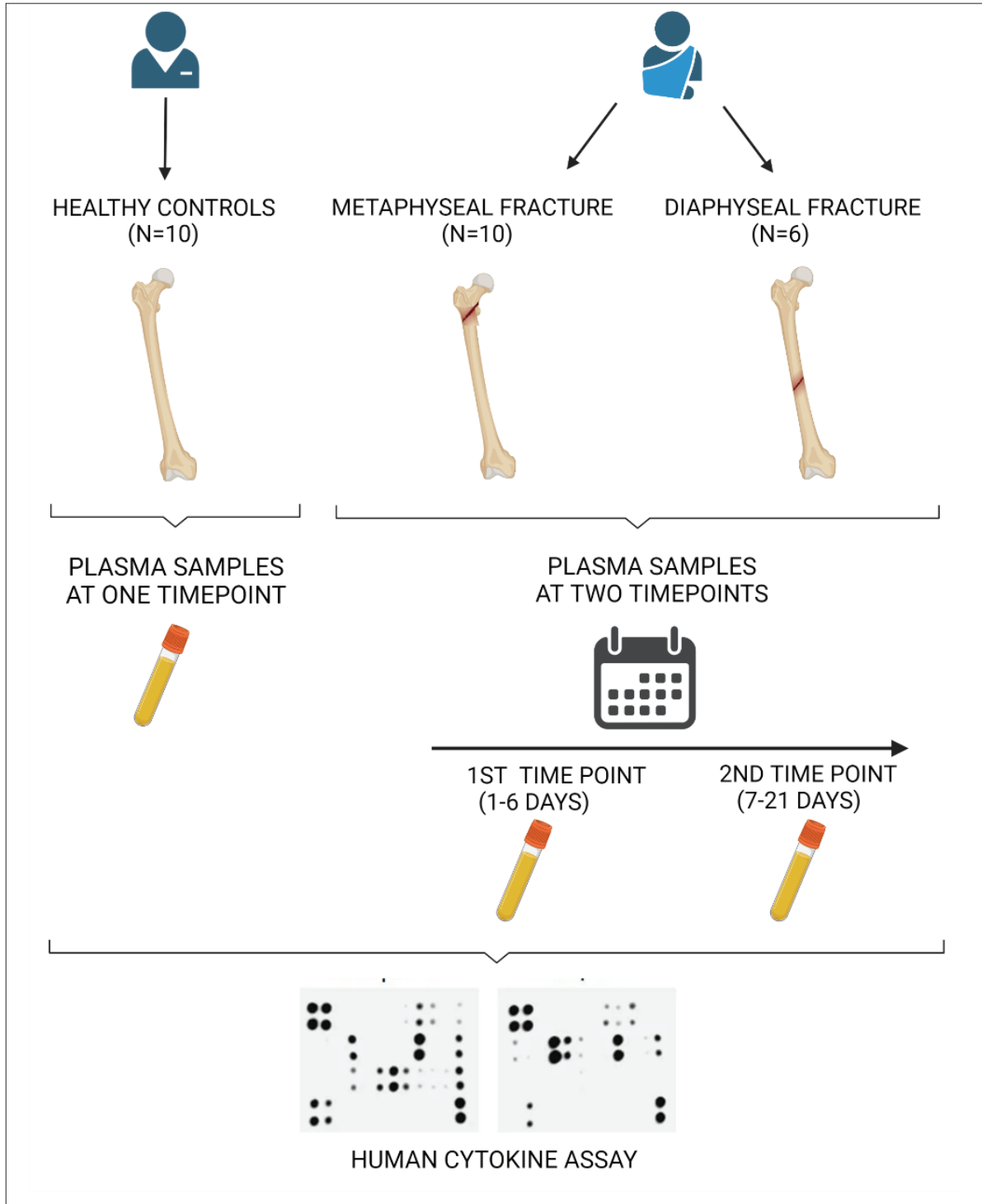
Figure 1. Study outline depicting subject groups, samples and methods.
Image created with BioRender.com.

Table 1. Inclusion and exclusion criteria employed to recruit study participants with bone fractures.

INCLUSION CRITERIA	EXCLUSION CRITERIA
Provided written consent	Malignant tumor
Age 18-65 years	Age over 65 years
Clinical and radiological verification of metaphyseal and diaphyseal fractures	Immunocompromised patients
Fracture of the radius, ulna, humerus, femur, tibia or fibula	Underlying osteoporosis
	Active infection

METHODS

Blood samples were drawn from participants by venipuncture and were stored into two 5 mL vacuette blood collection tubes containing 3.8% sodium citrate (blood to anticoagulant ratio 1:9). Plasma was isolated by centrifugation at 1.5 rcf for 15 minutes and were stored at -80 °C until further analysis.

The obtained plasma samples were pooled into 5 different experimental groups to be analysed using cytokine arrays. Experimental groups (sample pools) were the following: *i)* metaphyseal fractures at the first timepoint (N=10); *ii)* diaphyseal fractures at the first timepoint (N=6), *iii)* metaphyseal fractures at the second timepoint (N=10); *iv)* diaphyseal fractures at the second timepoint (N=6) and *v)* healthy controls (N=10). Pools were analyzed using the Human Cytokine Antibody Array – Membranes (Abcam, ab133996) according to manufacturer's instructions. Results were analyzed using the Chemi Doc imaging system (BioRad) according to manufacturer's recommendation. Background correction and quantification of fluorescent signals was performed with ImageJ 1.52a software (NIH). Expression of each cytokine was thus semi-quantitatively determined and reported for each experimental group as fold-change in comparison to the healthy control experimental group (which served as baseline). The heatmap of expression levels of cytokines was made using the matrix visualization and analysis software *Morpheus* (20).

RESULTS

Patients' characteristics are presented in Supplementary Table 1. The fold change relative to healthy controls for each of the 23 analysed plasma cytokines in each sample group is shown in Figure 3. Furthermore, numerical fold change results for all samples and cytokines are shown in Supplementary table S2.

Marked differences in cytokine expression were found between the fracture groups and the control group (Figure 3). By observing differences in general cytokine expression trends between groups, the majority (17 in the first and 20 in the second timepoint, respectively) of the analysed cytokines in the metaphyseal group show relative expression levels which are lower than those of the control group (Figure 3). Results from the diaphyseal groups are somewhat different, as only 8 cytokines from the first and second timepoint showed lower relative expression levels compared to the control group (Figure 3), while the majority of cytokines show expression levels lower than the baseline. The heatmap of the results shows that the significantly differentially expressed cytokines can effectively separate the comparison groups showing possible background occurrences in both types of healing (Figure 2). It is clearly observed that the diaphyseal group has an "activated" pro-inflammatory cytokine profile with markedly higher levels of cytokines at both timepoints compared to the metaphyseal group, which in contrast shows a much more muted, or even "silenced" cytokine expression profile.

Single cytokine analysis showed that in both metaphyseal and diaphyseal fracture groups the cytokines MCP-1 and RANTES showed the most prominent fold change at both timepoints. In the metaphyseal groups, increases in relative MCP-1 expression of 125% (first timepoint) and 107% (second timepoint)

were observed. Results from the diaphyseal group showed an increase in MCP-1 expression of 141% and 134% for the first and second timepoint, respectively (Supplementary table S2). Increase of RANTES expression was also markedly changed in all groups and ranged from 59% (first timepoint of metaphyseal group) and 80% (second timepoint diaphyseal group). IL-6 and TNF-α also show similarly elevated levels in both timepoints in the diaphyseal fracture group, whereas this is not observed in

the metaphyseal group. Furthermore, IL-3 expression was also elevated in the diaphyseal group, but only in the first timepoint (Figure 3). Additionally, GM-CSF expression levels were considerably lower from the baseline in both groups at both timepoints (Figure 3). It is also worth noting that MCP-3 expression levels in the metaphyseal group are the only to show a decrease in the expression levels greater than 30% at both timepoints (Supplementary table S2).
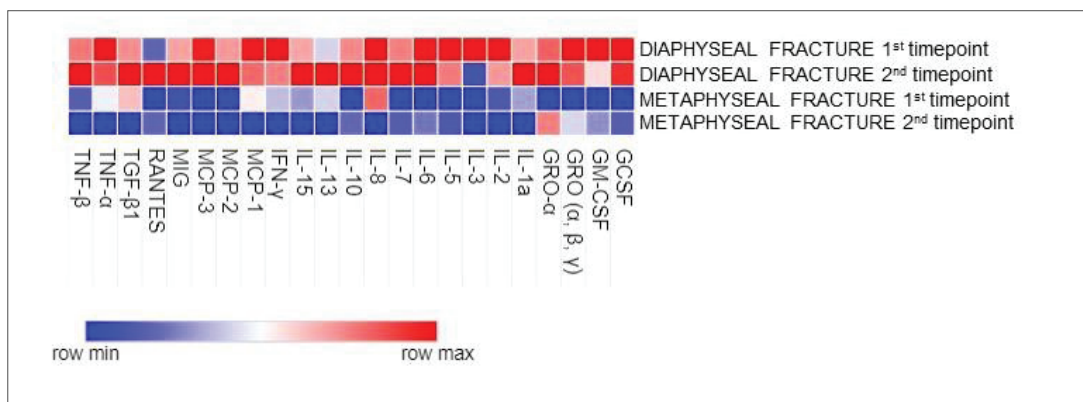

Figure 2. Heatmap showing expression levels of 23 cytokines from the diaphyseal and metaphyseal fracture groups. Red colour hues show higher, and blue hues show lower expression levels. A clearly visible trend of a pro-inflammatory pattern can be seen in the diaphyseal groups, whereas the cytokine response in the metaphyseal groups is much more muted.
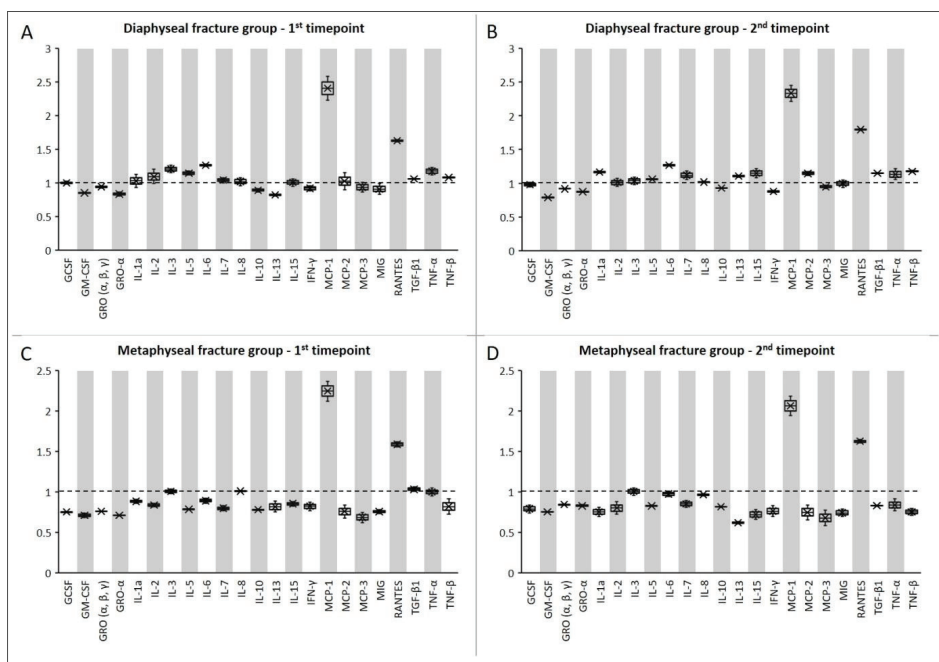

Figure 3. Graphs A - D show relative expression of analysed cytokines shown as fold change values (y-axis) of the diaphyseal (A and B) and metaphyseal (C and D) fracture groups compared to baseline values of the control group (dashed line).

DISCUSSION

Bone fracture healing is an extremely complex physiological mechanism which employs a plethora of factors including a vast array of different cell types which are tightly regulated through different mediators – namely, hormones, growth factors and cytokines, as well as other molecules. These mediators can either act locally in the tissue (paracrine signaling), or they can be plasma soluble; additionally, they could be transported with other modes of transportation, as protein cargo inside extracellular vesicles, whose role in bone healing is emerging (21). Since cytokines are crucial messengers that govern cell communication, which in turn enable basic cellular functions and coordination of multiple-cell actions; we focused on the systemic axis of bone fracture healing. By employing a cytokine array we intended to observe potential differences between metaphyseal and diaphyseal fracture, as well as to roughly estimate (using two time points) the existence of dynamic changes within each fracture healing group, and possibly, to capture the differential transition between the hematoma/inflammatory and the callus formation phase. Our study detected a distinct difference in cytokine profiles between metaphyseal and diaphyseal healing. A striking trend is visible in the metaphyseal group, where nearly all of the explored cytokines had lower plasma concentrations in comparison to healthy controls. Conversely, diaphyseal group showed an elevation of various proinflammatory cytokines (e.g. IL-1a, IL-6, TNF-α) as well as inflammatory markers at both timepoints. However, it is important to note that TGFβ, a classically pro-fibrotic, but pleiotropic cytokine, was also elevated. These findings indicate that diaphyseal fracture healing is driven by soluble component mediators, and that inflammation is the central mechanism involved in this type of healing. In contrast, inflammatory markers are "silenced" in patients with metaphyseal fractures. These observed differences between metaphyseal and diaphyseal fractures might be a result of their different mechanisms of MSC recruitment (22). As the metaphyseal region has more mesenchymal progenitors than the diaphyseal region, it might case that diaphyseal healing requires more pronounced cytokine signaling in order to regulate MSC recruitment and proliferation in the periosteum, as e.g. MCP-1 and TNF-α have been shown to do (23,24). The inflammatory phase of bone healing itself has been described to be limited, shorter and to terminate earlier in metaphyseal than diaphyseal fractures in animal models (22). This might explain our results in two ways, as they might be due to a similarly limited inflammatory phase in human fractures, as well as the fact that the inflammation might have been abrogated by the time samples were taken. The differing role of inflammation on fracture healing has also been shown by Sandberg et al., who demonstrated that downregulation of inflammatory response by glucocorticoids inhibits diaphyseal fracture healing, and in contrast increases the strength of regenerated metaphyseal bone (18).

A constant is visible in two cytokines among both types of healing, at both time points: two cytokines with emerging roles in bone healing – elevation of MCP-1 and RANTES. MCP-1 (also known as CCL-2) has an important role in osteoclastogenesis (and therefore bone remodeling) and has also been associated with injury-related heterotopic ossification (25). Furthermore, MCP-1 signaling has shown to be essential for the recruitment of MSCs during early stages of fracture healing in animals models (23). RANTES (CCL5) acts as a chemoattractant for immune cells as well as for osteoblasts and has previously been hypothesized to be a culprit-trigger molecule for the induction of heterotopic ossification in a rare bone disease – fibrodysplasia ossificans progressiva (FOP) (26,27). Moreover, increased RANTES expression was observed locally, at the site of hematoma formation as well as in the surrounding bone marrow in human specimens (27,28). To add, we found RANTES and MCP-1 to be elevated in post-COVID exacerbation of heterotopic ossification in FOP patient (29). Our findings, taken together with this theoretical background, could imply that MCP-1 and RANTES might be constitutional chemoattractant drivers of bone healing and formation, especially in early phases, irrespective of fracture type. It is important to take into account that most patients with diaphyseal, and some with metaphyseal fractures underwent surgical treatment, which is known to induce an inflammatory response (30). However, we believe that, the distinction between metaphyseal and diaphyseal groups is not solely the result of surgical inflammatory stimulus, as this distinction wouldn't be as apparent at both time points. Rather, the "chasm" between the groups would probably reduce at the second time point, which was predominately weeks after surgery.

In order to draw valid conclusions, the limitations of this study must be taken into account. First, this study is limited by the small sample size and by employing only two time points where each spans over the period of several days. Second, samples were pooled into experimental groups which made it impossible to study individual pateient differences in bone healing mechanisms. However, by pooling the samples, we were able to get a "biological average". Third, as we mentioned, it is important to take into account the fact that some patients underwent surgery, and others didn't. Fourth, our baseline were healthy female volunteers, whereas fracture patients were both male and female. Fifth, cytokines were analysed semi-quantitatively.

However, we believe that in spite of these limitations, our pilot study indicated chemokines which might be potential crucial drivers of bone healing, as well as painted distinct cytokine plasma profiles of metaphyseal and diaphyseal healing - and therefore shed further light on the fact that these types of fractures heal by employing different mechanisms. As cytokines likely present some of the many cogwheels which move these processes, this topic demands further research in order to fully understand them and consequently improve treatment modalities and potentially patient outcomes.
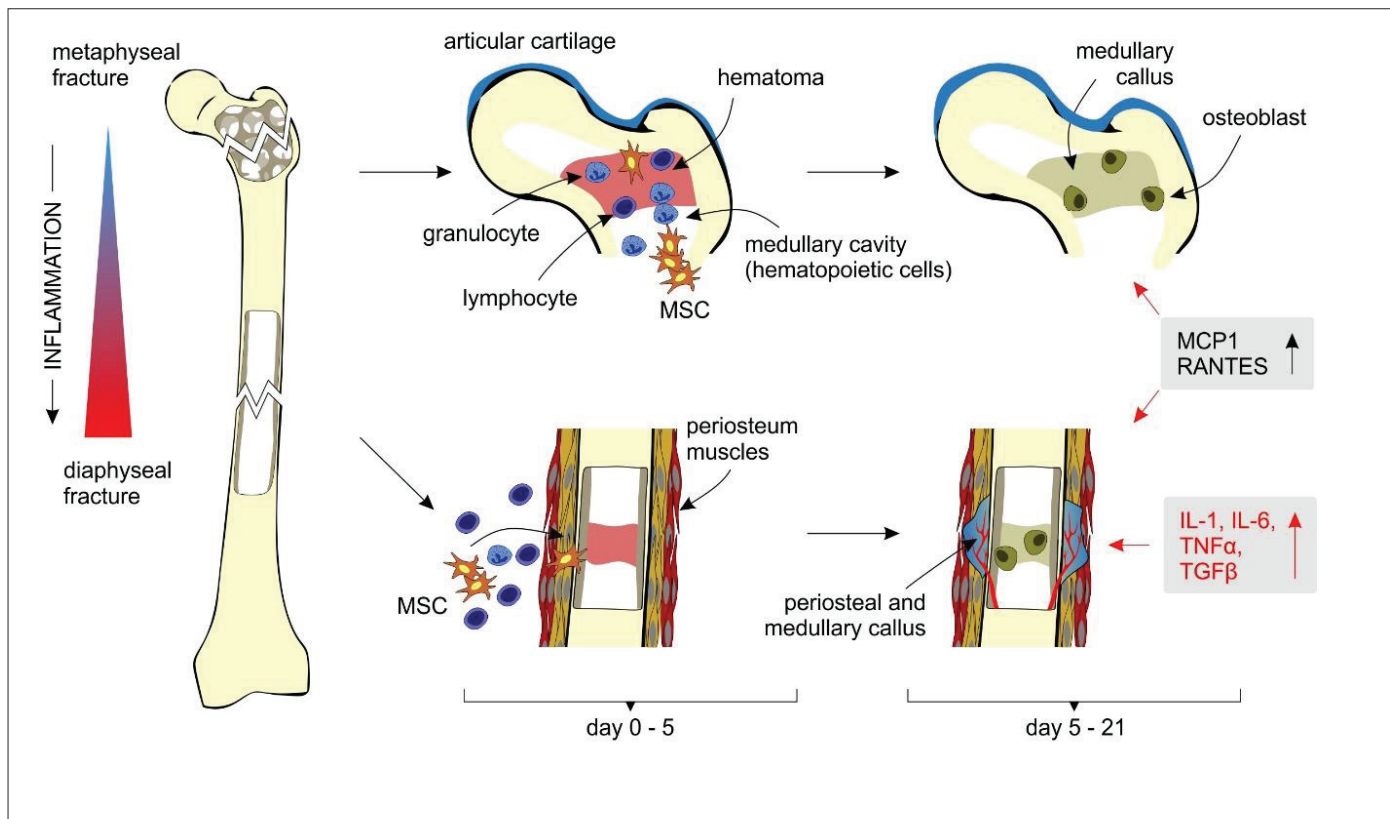
Figure 4. Illustration depicting differences between metaphyseal and diaphyseal fracture healing. Metaphyseal fractures result in predominantly bone marrow derived stem cell migration into the fracture site and differentiation into bone derived osteoblasts. Both proinflammatory cytokine and inflammatory cell levels are suppressed, apart from RANTES and MCP 1. In contrast, diaphyseal fractures result in predominantly periosteal derived stem cell migration and differentiation. Both proinflammatory cytokine and immune cell levels are upregulated compared to metaphyseal fractures.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by the Hospital Centre Sisters of Charity's Ethical Committee (EP-003-06/20-03/023).