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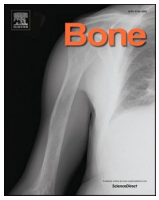

Review Article

Is there a role for N1-N2 neutrophil phenotypes in bone regeneration? A systematic review

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ABSTRACT

Purpose: This review aims to provide an overview of the multiple functions of neutrophils, with the recognition of the inflammatory (N1) and regenerative (N2) phenotypes, in relation to fracture healing.

Methods: A literature search was performed using the PubMed database. The quality of the articles was evaluated using critical appraisal checklists.

Results: Thirty one studies were included in this review. These studies consistently support that neutrophils exert both beneficial and detrimental effects on bone regeneration, influenced by Tumor Necrosis Factor-α (TNF-α), Interleukin 8 (IL-8), mast cells, and macrophages. The N2 phenotype has recently emerged as one promoter of bone healing. The N1 phenotype has progressively been connected with inflammatory neutrophils during fracture healing.

Conclusions: This review has pinpointed various aspects and mechanisms of neutrophil influence on bone healing. The recognition of N1 and N2 neutrophil phenotypes potentially shed new light on the dynamic shifts taking place within the Fracture Hematoma (FH).

1. Introduction

Neutrophils account for 60–70 % of white blood cells in humans and play a pivotal role in the innate immune system [1–3]. Neutrophils serve as a crucial frontline defense mechanism, being activated by both Pathogen-Associated Molecular Patterns (PAMPs) and Damage-Associated Molecular Patterns (DAMPs) [4]. The mode of action of neutrophils is complex, including deformation, adhesion, infiltration, and migration to support neutrophil infiltrate into the inflammatory site [5,6]. After engulfing PAMPs and DAMPs [7], neutrophils interact with other innate and adaptive immune cells [7,8], form Neutrophil Extracellular Traps (NETs) [9], and produce Reactive Oxygen Species (ROS), cytokines and granular proteins [10,11]. By doing so, neutrophils can respond effectively to signals that indicate potential harmful conditions in the human body.

Although these cellular mechanisms are very effective, they can also cause abundant inflammation due to an imbalanced immune response, for example in severe trauma. Exacerbated neutrophil activation can contribute to the occurrence and progression of conditions like Acute Respiratory Distress Syndrome (ARDS) and Systemic Inflammatory Response Syndrome (SIRS) [12]. A balanced inflammatory response is required for tissue regeneration, but prolonged or abundant inflammation due to neutrophil activity can inhibit tissue repair. One example of this is shown in the osteogenic differentiation of Bone Mesenchymal Stem Cells (BMSCs) in the presence of an excessive number of neutrophils, which has proven to impair extracellular matrix (ECM) mineralization [13].

It is unclear at this point whether the role of neutrophils in tissue regeneration can only be attributed to one single phenotype of neutrophils [14]. In addition to the classical involvement of neutrophils in the inflammatory response, research has broadly shown that neutrophils also exhibit regenerative characteristics [15–17], suggesting a similarity with the different behaviors of M1 and M2 phenotype macrophages. In fact, different neutrophil phenotypes have been identified as N1 and N2 neutrophils, with a role in inflammation and regeneration, respectively [17]. Various surface biomarkers have been used to identify N1 and N2

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phenotypes. Higher expression of CD54 [18–20] and CD95 [18] are associated with N1 phenotype, and CD182 [18,20], CD184 [19] and C206 [21–24] with N2 phenotype, although the expression patterns show variation between studies. Ohms et al. successfully induced the polarization of human neutrophils into N1 and N2 phenotypes *in vitro*, utilizing CD54 and CD95 as N1 biomarkers, and CD182 for N2 [18]. Subsequent studies have consistently relied on these surface biomarkers to distinguish between N1 and N2 phenotypes [19,20].

Fracture healing is a key example of tissue repair in which neutrophils are involved. Neutrophils are among the first cells to arrive at the fracture site, regulate the inflammatory response, and initiate bone regenerative processes [25]. It is well-established that neutrophils contribute to clearing DAMPs and PAMPs, forming NETs, as well as releasing ROS, neutrophil serine proteases, and various cytokines, including TNF-α, IL-1b, IL-6, IL-10, and Monocyte Chemotactic Protein-1 (MCP1), at the fracture site [26,27]. These cytokines play a role in facilitating interactions between neutrophils and other cells, such as mast cells and macrophages [26]. Furthermore, neutrophils actively contribute to angiogenesis by secreting Vascular Endothelial Growth Factor (VEGF) and participate in fibronectin formation, aiding in stromal cell recruitment at the fracture site to promote bone healing [28,29]. These mechanisms of neutrophils in fracture healing underscore the importance of maintaining a balance between pro-inflammatory and anti-inflammatory responses. In a recent review by Kovtun et al., the dual effects of neutrophils on fracture healing were described in detail [14]. However, the potential role of the newly discovered N1-N2 neutrophil phenotypes in bone regeneration remains unexplored. Our hypothesis is that the different neutrophil phenotypes, N1 and N2, have distinct contributions to bone repair. The aim of this systematic review is therefore to provide an overview on the role of neutrophils in bone regeneration, and to identify phenomena in the bone healing process that could be attributed to either N1 or N2 phenotypes.

2. Methods

2.1. Literature search

The literature review was performed based on the principles described in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [30]. A search was conducted in the PubMed database using the search terms (*neutrophil*) *AND* ((*bone regeneration*) *OR* (*fracture healing*)), followed by applying the filters "*English*" and "*10 years*". All retrieved articles were filtered and selected based on the relevance of each title and abstract by two independent researchers (FL and SV) using the following criteria:

Exclusion criteria:

i. Regeneration of other tissues than bone
ii. No description of the role of neutrophils in relation to bone
iii. Limited to the interactions between materials and neutrophils
iv. Review articles, case-reports, and meta-analyses

For all remaining articles, the full text was read by two independent researchers (FL and SV), and the articles were filtered using the same criteria mentioned above. Included articles were categorized as pre-clinical *in vitro* studies, *in vivo* studies, or retrospective clinical studies.

2.2. Quality assessment

Two critical appraisal guidelines were used by two independent researchers (FL and SV) to evaluate the quality of the selected articles:

i. ARRIVE 2.0 Checklist for the pre-clinical *in vivo* articles [31]
ii. NIH Quality Assessment of Case-Control Studies Tool for retrospective clinical research articles [32]

The ARRIVE Guidelines 2.0 Checklist Tool consists of 38 specific questions to systematically dissect the quality and validity of an article. A score of less than 50 % is categorized as poor quality, 50 %–80 % is considered average quality, and a score exceeding 80 % is recognized as excellent quality. The NIH Quality Assessment of Case-Control Studies Tool consists of 12 specific questions to evaluate the quality of retrospective clinical studies. An article is deemed acceptable if it attains a score of 60 %. To ensure uniformity and standardization across various checklists, a minimum of 60 % was used as a threshold for the inclusion of *in vivo* and retrospective clinical studies in this review [33,34].

3. Results

3.1. Search results

The initial search in the PubMed database yielded 420 articles. Applying the filter settings "English" and "10 years" resulted in 204 remaining articles. After screening the title and abstract of these publications, 33 articles remained. None of these were excluded after full text reading. The 33 articles comprised of pre-clinical *in vivo* studies ($n = 26$), retrospective clinical studies ($n = 2$), and pre-clinical *in vitro* studies ($n = 5$). The quality of the pre-clinical *in vivo* studies and retrospective clinical studies was assessed using the ARRIVE 2.0 Checklist and the NIH Quality Assessment of Case-Control Studies Tool, respectively (Supplement 1 and 2). All scores, percentages, and scoring overviews are displayed in Table 1, and Supplement 3 and 4. Two pre-clinical *in vivo* studies had percentages below the 60 % threshold and were excluded. In total, 31 articles were included in this systematic review. A flow chart, based on the PRISMA 2020 flow diagram [30], summarizing the search results including the number of articles at each stage of the review process is shown in Fig. 1.

From the retrieved articles, 24 studies were performed *in vivo* studies, five articles used *in vitro* models, and two articles analyzed retrospective clinical data. The 24 *in vivo* studies consisted of 19 rodent models (14 mouse models and five rat models), two human samples, one rabbit model, one porcine model, and one study that used both mouse model and human sample (Fig. 2). In the five *in vitro* studies, one study used HL-60 cells and human Saos-2 cells. The remaining four studies all used human neutrophils of healthy volunteers. Additionally, one study involved human BMSCs, one involved human umbilical vein endothelial cells (HUVECs) combined with human osteoblasts (HOBs), and one involved human SCP-1 cells. The two retrospective clinical studies investigated the relationship between the number of circulating neutrophils and the outcome of fracture healing.

The included studies were discussed in two categories: "neutrophils in bone regeneration" and "neutrophil responses to cytokines and interactions with other cell types in bone regeneration".

3.2. Neutrophils in bone regeneration

Although the role of neutrophils in relation to bone regeneration has been the topic of many studies, the results are often ambiguous. Several studies described a positive role of neutrophils in bone regeneration [28,29,35,36], while others came to a negative role [13,37–39]. Clinical studies have clearly demonstrated the infiltration of neutrophils from peripheral blood into the FH within hours after the fracture occurred [40,41], but the role and influence of neutrophils in the FH remain unclear. Their presence in the FH has been associated with early ECM formation, but a decreased amount of mineralized ECM [13,28,29,35]. Recent studies aimed to connect the heterogeneity of neutrophil phenotypes with the different effects of neutrophils on bone regeneration [35], aligning with the recognition that N1 or N2 phenotype plays an inflammatory or regenerative role as mentioned above [17]. Specifically, the recent identification of regenerative N2 phenotypes provides novel perspectives into the positive effects of neutrophils on bone regeneration [35].

Table 1
In vivo studies and retrospective clinical research critical appraisal scores and percentages.

In vivo study	Score (total score) and percentage
Shimoide et al.-2018 [44]	29 (38), 76 %
Liu et al.-2021 [57]	27 (38), 71 %
Cai et al.-2021 [35]	28 (38), 74 %
Greven et al.-2020 [39]	32 (38), 84 %
Herath et al.-2021 [36]	31 (38), 82 %
Haffner-Luntzer et al.-2019 [48]	30 (38), 79 %
Fischer et al.-2021 [60]	29 (38), 76 %
Rana et al.-2021 [62]	34 (38), 89 %
Buren et al.-2018 [43]	33 (38), 87 %
Gao et al.-2013 [92]	22 (38), 58%[b]
Dishowitz et al.-2013 [38]	28 (38), 74 %
Meesters et al.-2016 [37]	30 (38), 79 %
Kovtun et al.-2016 [27]	26 (38), 68 %
Forster et al.-2016 [58]	28 (38), 74 %
Bastian et al.-2016 [29]	27 (34), 79 %[a]
Chan et al.-2015 [56]	29 (38), 76 %
Recknagel et al.-2013 [47]	27 (38), 71 %
Lu et al.-2013 [93]	20 (38), 53%[b]
Liu et al.-2022 [46]	23 (34), 68 %[a]
Zhu et al.-2023 [45]	28 (38), 74 %
Kuhn et al.-2022 [50]	31 (38), 82 %
Zhang et al.-2022 [42]	26 (38), 68 %
Tschaffon-Müller et al.-2023 [49]	26 (38), 68 %
Liu et al.-2022 [63]	26 (38), 68 %
Li et al.-2022 [53]	23 (38), 61 %
Li et al.-2023 [55]	27 (38), 71 %

Retrospective clinical study	Score (total score) and percentage
Hesselink et al.-2018 [41]	10 (12), 83 %
Bastian et al.-2016 [40]	11 (12), 92 %

[a] Human samples: four out of 38 questions did not apply for human samples based on the ARRIVA 2.0 Checklist.
[b] Articles with a percentage less than 60 % were excluded.

Three studies have shown that neutrophils at the injury site are beneficial for bone repair. Herath et al. demonstrated that injecting neutrophils in a murine cranial bone defect leads to a faster bone regeneration [36]. Furthermore, Cai et al. demonstrated that the type of neutrophils to promote bone regeneration is the N2 neutrophil, which can be attracted to the fracture site by IL-8 in murine models [35]. More specifically, N2 neutrophils secrete Stromal Cell-Derived Factor-1α (SDF-1α) to attract BMSCs *via* the SDF-1/C-X-C Motif Chemokine Receptor 4 (CXCR4) axis and its downstream Phosphatidylinositol 3′-Kinase (PI3K)/Protein Kinase B (Akt) pathway, as well as β-catenin mediated migration. Furthermore, this study proposed that Transforming Growth Factor Beta 1 (TGF-β1) plays a role in the conversion of N1 phenotype to N2 phenotype neutrophils [35]. Although TGF-β1 may play a role in promoting fracture healing by attracting BMSCs to the fracture site, it remains uncertain whether neutrophil phenotype conversion is the mechanism involved. Additionally, the positive effects of neutrophils are also influenced by the age of the host. In mouse models, Zhang et al. demonstrated that implanting neutrophils from a younger mouse into an aged mouse can enhance fracture healing through the expression of Extracellular Vesicles (EVs) [42].

Neutrophils have also been shown to promote angiogenesis, which is a prerequisite for fracture healing. One *in vitro* study described the comparison of angiogenic and osteogenic gene expression in a co-culture of human neutrophils, HOBs, and HUVECs with a co-culture of HOBs and HUVECs [28]. In this study, neutrophils stimulated a higher expression of typical angiogenic and osteogenic markers, such as VEGF-A, CD34, Fibroblast Growth Factor-2 (FGF-2), Alkaline Phosphatase (ALP), Type I Collagen (COL-1), Osteopontin (OPN), and Osteocalcin (OCN) [28]. Therefore, neutrophils have been proven to benefit fracture healing both *in vivo* and *in vitro*.

Neutrophils can influence the ECM formation during the inflammatory phase of bone healing [13,29]. The infiltration of human neutrophils into the FH during the first two days after fracture has been associated with the synthesis of fibronectin containing ECM, as a first step in bone regeneration *in vivo* [29]. On the other hand, neutrophils can decrease the amount of mineralized ECM and thereby impair bone regeneration. Bastian et al. illustrated a parabolic relationship between the number of neutrophils and the ECM mineralization by human BMSCs. They observed a decrease in ECM mineralization in the presence of both high and low numbers of neutrophils [13]. Overall, these studies indicate that neutrophils do not only stimulate the ECM formation but also inhibit the ECM mineralization in a concentration-dependent way.

This concentration-dependent influence of neutrophils on bone healing is supported by other studies as well, which showed that an excessive number of neutrophils at the fracture site impairs bone regeneration. For example, Nos2−/− and Nos3−/− mice displayed a lower amino acid concentrations accompanied with an elevated neutrophil infiltrate with a higher level of Myeloperoxidase (MPO) in the callus at 28 days after fracture, compared to wild type mice. Eventually, both disturbed amino acid metabolism and the prolonged increase of neutrophil infiltration led to bone nonunion in the knockout mice [37]. In another example in murine models, inhibiting Notch signaling before a fracture stimulated higher levels of inflammatory cytokines, such as TNF-α and IL-1β. Subsequently, an excessive number of neutrophils were attracted by these cytokines, resulting in a larger void space in the callus at 10 days after fracture. Additionally, osteogenic differentiation of BMSCs and subsequent mineralization were both impaired *in vitro* at two weeks [38].

The neutrophil count or function can be influenced by systemic diseases, such as osteitis, diabetes, severe trauma, and mental stress, and hence could alter the outcome of bone healing. However, the neutrophil-related effect on the bone formation is unclear, and several studies

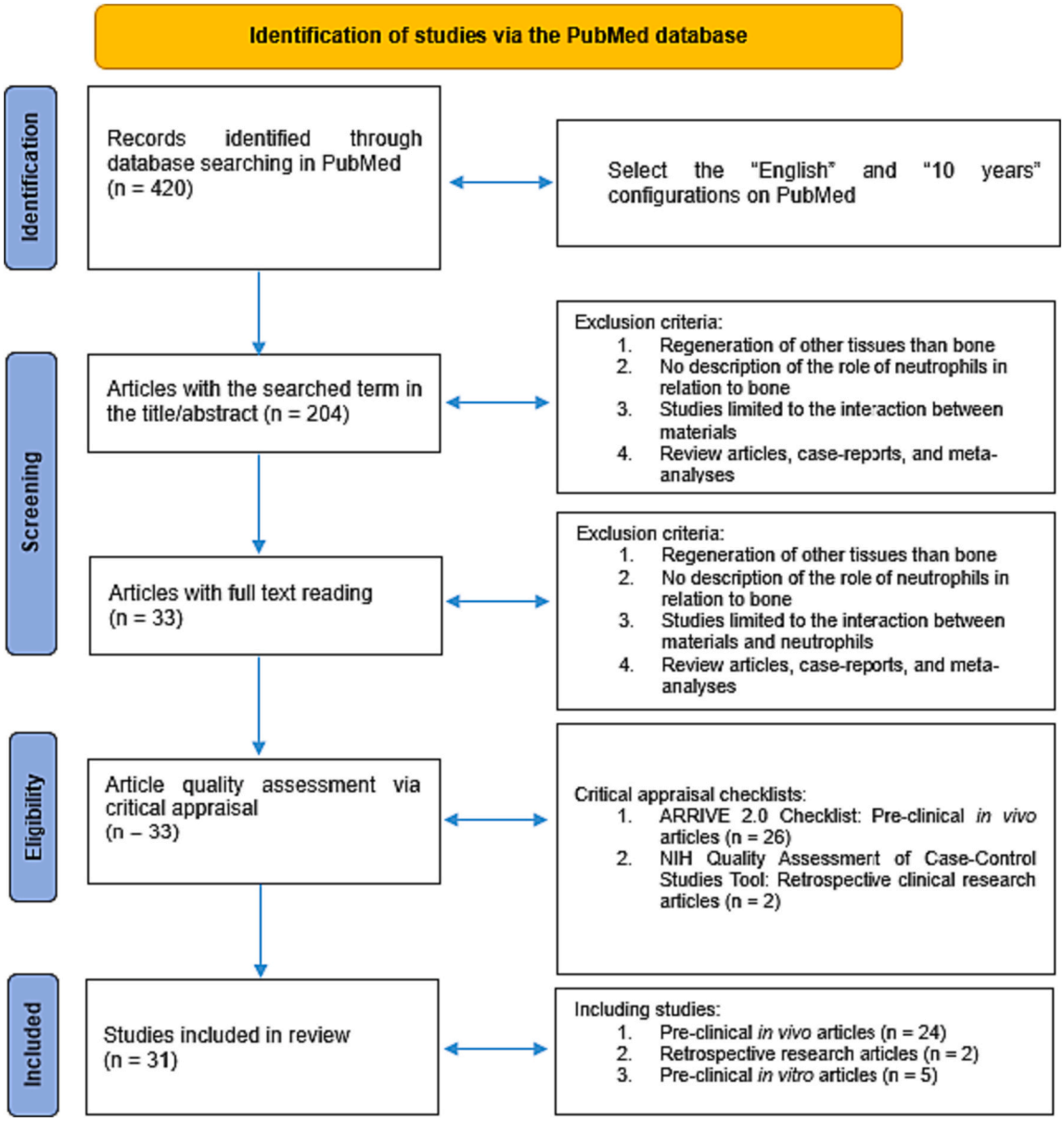

Fig. 1. Flow chart summarizing the literature search and selection process.

described contradictory results. Some studies investigating the effect of osteitis and diabetes on bone healing reported no significant alteration in the number of neutrophils present at the fracture site and hence no effect on the outcome of bone healing [43,44]. In the case of diabetes, however, one study indicated that hyperglycemia could induce the formation of NETs, leading to an excessive inflammatory response and consequently inhibiting osteogenesis *in vitro* [45]. Through an investigation using diabetic rat models, this study also demonstrated that the negative impact of NETs on bone healing can be alleviated by metformin [45]. In humans, another study has shown that in fracture patients with diabetes, neutrophil activation can be mediated by up-regulated ANXA3, resulting in fracture non-union [46]. Taken together, diabetes may impede bone regeneration by influencing the function of neutrophils rather than their quantity.

After severe trauma, and during mental stress, two studies reported a negative effect on bone healing due to an increased infiltration of neutrophils [47,48]. In the latter study, the β-adrenoreceptor signaling was identified as the responsible pathway for the increased infiltration of neutrophils in the FH [48]. Additionally, Tschaffon-Müller et al. demonstrated that mental stress in fracture patients can elevate the level of Tyrosine Hydroxylase (TH) in the FH. Subsequently, TH can induce neutrophils to express catecholamines to inhibit chondrocytes, which is in concert with β2-adrenoceptor signaling in chondrocytes, resulting in impaired bone healing [49]. Kuhn et al. have shown that by performing knockout of TH in mouse models, the number of neutrophils is increased, leading to excessive inflammation at three days post-fracture, which partially contributes to impaired bone healing on day 21 [50].

Neutrophils, through the formation of NETs, can exert an influence on bone healing [51–53]. However, the impact of NETs on bone healing remains ambiguous. In addition to the negative role of NETs observed in diabetic rat models, as described above [45], two studies have consistently suggested that NETs play an adverse role in bone healing [51,52]. Specifically, one *in vitro* study has demonstrated that NETs can impede the migration and differentiation of MSCs, ultimately leading to cell death and impaired osteogenesis [51]. Another study showed the negative effect of NETs formation on bone healing, which can be mitigated by low-frequency pulsed electromagnetic fields, without affecting Ca2+ influx or ROS formation [52]. Conversely, a separate study

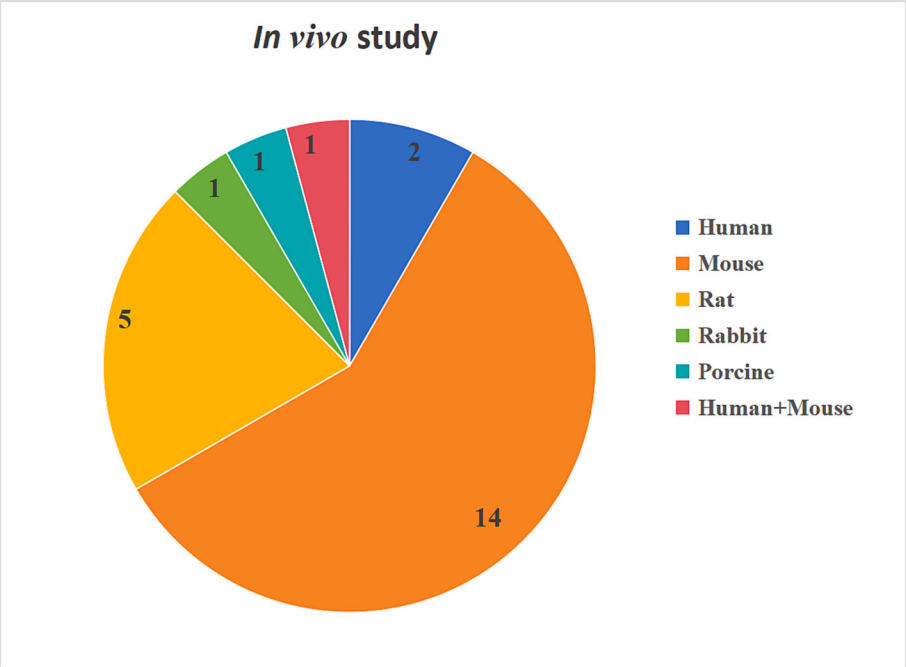

Fig. 2. *In vivo* study sample.

revealed that NETs formation can be enhanced by the implantation of zinc-doped ferric oxyhydroxide nano-layer scaffold, which activates the NOX/ROS signaling pathway in neutrophils, subsequently promoting osseointegration [53].

Neutrophils can be influenced by (metallic) implants, thereby affecting osseointegration. However, the exact nature of this influence remains unclear. One study has demonstrated that neutrophils can hinder titanium osseointegration by impeding osteoblast attachment and function [54]. Conversely, another study demonstrated that mature G3 neutrophils can encapsulate an implanted strontium-modified titanium scaffold, subsequently recruiting BMSCs through the CXCL12/CXCR3 signaling axis, ultimately promoting bone healing [55]. The identification of the CXCL12/CXCR3 pathway in neutrophil recruitment of BMSCs is consistent with the findings in the study by Cai et al., as mentioned above [35].

In addition to the effect of local neutrophils on fracture healing, there is a clear relation between the number of circulating neutrophils and bone regeneration as described in several studies [27,40,41]. Depletion of circulating neutrophils resulted in impaired bone healing at 21 days after fracture in mice [27]. Other studies have shown that the dynamics of circulating neutrophils are associated with different fracture healing outcomes. Two retrospective clinical studies investigating isolated fractures have revealed that reduced counts of circulating neutrophils during the early stage of fracture healing are associated with inferior fracture healing outcomes. In comparison to patients with impaired fracture healing, those with normal fracture healing exhibit higher numbers of circulating neutrophils during the initial stage of the fracture healing cascade [40,41].

Taken together, these studies illustrated that the number of neutrophils is important for a balanced inflammatory response as well as the subsequent initiation and prolongation of fracture healing. An excessive presence of neutrophils, both locally at the fracture site and in the systemic circulation, can be detrimental to the proper initiation of the fracture healing cascade. On the other hand, neutrophil depletion at the fracture site or in the systemic circulation can lead to impaired fracture healing as well. An appropriate neutrophil infiltration in the beginning stage contributes to bone healing, whereas a prolonged neutrophil infiltration in the later stages is able to impair bone healing. This concentration-temporal-dependent relation between the number of neutrophils and fracture healing could very well explain the different outcome of fracture healing in mono-trauma and multi-trauma.

3.3. *Neutrophil responses to cytokines and interactions with other cell types in bone regeneration*

Several cytokines have been shown to participate in the inflammatory response, including IL-8 and TNF-α [35,38,56–58]. Although IL-8 has been described to attract the N2 neutrophils at the later stage of inflammation [35], the precise mechanism underlying the interplay between IL-8 and N1-N2 neutrophil phenotypes remains unclear. With respect to TNF-α, it has the capability to recruit neutrophils to the fracture site, resulting in a prolonged inflammatory response [38]. However, the impact of the recruited neutrophils remains ambiguous and is the subject of conflicting findings [38,56]. While these mechanisms have undergone scrutiny in the context of neutrophil migration, inflammation, surface receptors, and products, their connection to N1-N2 neutrophil phenotypes remains ambiguous [56–60].

IL-8 is an important cytokine in the neutrophil attraction and activation. One study showed that inhibiting IL-8 high-affinity chemokine receptor-related genes, such as CXCR1/CXCR2/C-C Chemokine Receptor Type 1 (CCR1), resulted in down-regulation of the Phospho-C-Jun N-terminal Kinase (p-JNK) in the Mitogen-Activated Protein Kinase (MAPK) signaling cascade [57]. Subsequently, the expression of several genes was reduced, such as the respiratory burst-related gene Cytochrome B-245 Beta Chain (CYBB), the inflammation-related gene Neutrophil Cytosolic Factor 2 (NCF2), and the Matrix Metalloproteinase-9 (MMP-9) gene. In this way, neutrophil chemotaxis and respiratory burst were reduced, resulting in less alveolar bone resorption in periodontal tissues. The present study illustrated that inhibiting IL-8, which is responsible for attracting neutrophils, can be beneficial for bone regeneration [57]. This is in contrast to the positive impact of IL-8-mediated recruitment of neutrophils on bone repair as mentioned above [35]. Taken together, these studies provide evidence that IL-8 plays a critical role in attracting neutrophils to the fracture site and influencing bone regeneration with potentially divergent outcomes.

Apart from IL-8, IL-8 homologues can attract more neutrophils to the

bone defect site during bone healing. One study demonstrated that in rat models, increased IL-8 homologues CXCL1, CXCL2, and CXCL3 were expressed in the dialysate from the bone defect, compared to the dialysate from the soft tissue wound. More neutrophils were attracted to wound fluids from bone defects, accompanied by more specific products promoting the action of ROS, such as Neutrophil Elastase 2 (NE-2), MMP-8, proteinase 3, and cathepsin G [58].

TNF is a typical pro-inflammatory cytokine, which promotes neutrophil-mediated inflammatory response in bone regeneration [38]. However, the effect of TNF-attracted neutrophils on bone regeneration has dual sides. In addition to impairing bone regeneration by increasing local neutrophilic inflammation and TNF-α expression [38], one study demonstrated that inhibition of TNF can reduce neutrophil counts, ultimately leading to impaired callus mineralization and osteogenesis at 28 days after fracture in murine models [56]. Contrarily, applying low-dose Recombinant Human TNF (rhTNF) to the fracture site within one day after fracture has been shown to promote neutrophil infiltration and CCL2 expression in murine models, which are critical for monocyte recruitment to promote bone regeneration [56]. These studies demonstrated that TNF increased the number of neutrophils at the fracture site, but the role of neutrophils in subsequent bone regeneration was conflicting.

In addition to responding to cytokines, neutrophils have the capacity to interact with other cell types, including mast cells and monocytes/macrophages, and can therefore influence downstream cascades in bone healing [56,60–62]. Neutrophils interact with other cells in a concentration-dependent manner, resulting in an increased infiltration of neutrophils. In ovariectomy mouse models, Fischer et al. demonstrated that mast cells can release several inflammatory cytokines, including IL-6, midkine, and CXCL10, which enhance neutrophil infiltration in the FH. Consequently, this excessive neutrophilic inflammation impairs bone regeneration [60]. Regarding macrophages, neutrophils produce CCL2, which contributes to the recruitment of more macrophages, ultimately initiating the process of bone regeneration [56,61,62]. One study also showed that the hybrid biomaterial (Gel@fMLP/SiO2-FasL) can manipulate the recruitment and apoptosis of neutrophils, and subsequently neutrophil engulfed by macrophages to initiate their phenotypic transformation, resulting in promoting bone regeneration [63]. However, these macrophages, which are beneficial for bone regeneration, were not clearly linked with the M2 phenotype [56]. While one study endeavored to elucidate the interplay between N1-N2 neutrophil phenotypes and M1-M2 macrophage phenotypes, the results remain inconclusive [35].

In summary, the current literature suggests that TNF-α, IL-8, mast cells, and macrophages play roles in regulating neutrophil activity, leading to either a stimulatory or an inhibitory effect on bone regeneration. Furthermore, neutrophils have the potential to influence the downstream cascade of M1-M2 macrophages, albeit the underlying mechanism is yet unclear. The N2 neutrophil was initially described in the context of bone regeneration, which can be attracted to the fracture site by IL-8, thereby promoting bone healing. While current studies have not yet established a clear link between the inflammatory neutrophil and the N1 phenotype neutrophil, the role of N1 neutrophils in bone regeneration and their response to pro-inflammatory cytokines have garnered attention. It is crucial to ascertain the response of neutrophils to cytokines and their interplay with other cell types, particularly the contribution of the N1-N2 neutrophil phenotypes in driving macrophage polarization towards the M2 phenotype, to obtain a better comprehension of the interplay between the immune response and fracture healing.

4. Discussion

Several studies indicated an ambiguous role for neutrophils in bone regeneration [27–29,36,56,64,65]. The contradictory effects of neutrophils have been shown in different stages of bone regeneration and at different levels, for example in the interaction with other cells or the response to cytokines. We hypothesize that the identification of N1 and N2 neutrophil phenotypes could at least in part explain the two-sided effects that neutrophils appear to have on bone regeneration. Therefore, this review aims to focus on the intricate roles of neutrophils in bone regeneration, exploring their potential relationship with different neutrophil phenotypes. Although the amount of evidence is still limited, there appears to be a clear role for N1 and N2 neutrophil phenotypes at different stages of fracture healing, and they express different cytokines, display different responses to cytokines, and have different interactions with other cells.

To initiate fracture healing, the formation of the FH takes place initially. The environment associated with FH is characterized by low pH, hypoxia, high lactate levels, and elevated concentrations of inflammatory cytokines that attract immune cells. Notably, these conditions are reminiscent of the tumor environment [18,66,67], where the original identification of N1 and N2 neutrophils was described by Fridlender et al [17]. However, the dynamics of neutrophils during fracture healing are different from the tumor environment, as neutrophils are drawn to the fracture site to aid in debris clearance and initiate the repair process [14]. Whether this involves N1 or N2 neutrophil phenotype is unclear, even though different cytokines are expressed by each of them [18,35,38,56–58]. TNF-α is mainly expressed by N1 neutrophils compared to N2 neutrophils [18,68], leading to neutrophil recruitment during the early stage of fracture healing [38,56,69,70]. At a later stage, the levels of IL-8, which is also responsible for neutrophil recruitment, increase in the FH, but IL-8 is mainly expressed by N2 neutrophils [18]. The temporal change in expression of TNF-α and IL-8 is therefore an indicator of a change in the neutrophil population, shifting from the inflammatory N1 to the regenerative N2 phenotype.

N1 neutrophils can convert to N2 and *vice versa* under the influence of different stimuli [17,18,71]. To convert neutrophil phenotypes, TGF-β1 is a potent convertor of both unstimulated neutrophils (N0) and N1 neutrophils to the N2 phenotype, and several other anti-inflammatory cytokines were shown to have the same effect [17,18]. During fracture healing, TGF-β1 is highly expressed in the FH [72] and thereby promotes osteoprogenitor cell attraction [73] as well as the osteogenic capacities of osteoblasts [74]. The simultaneous conversion of N1 neutrophils to N2 neutrophils by TGF-β1 would contribute to the regenerative environment that is required in the FH. Gradual conversion of the neutrophil population from N1 to N2 under the influence of TGF-β1 is a likely mechanism to achieve this.

Neutrophils interact with other cell types during fracture healing. For example, neutrophils and macrophages interact with each other to influence downstream bone healing processes. During fracture healing, N1 neutrophils attract monocytes by secreting MMPs, monocyte chemoattractant proteins, and macrophage inflammatory proteins [75–77]. Stimulated by these inflammatory cytokines, monocytes convert to the inflammatory macrophage phenotype (M1) [78]. However, the influence of neutrophils on subsequent conversion of macrophages to the regenerative phenotype (M2) is not clear. Several studies showed that N1 neutrophils and tissue debris contribute to the M2 formation [56,62], whereas one other study identified N2 neutrophils as the inductors of M2 polarization [35]. In addition to interacting with macrophages, this review also demonstrated that mast cells can induce neutrophil activation through the expression of IL-6, leading to an inflammatory response in the early stages of healing [60]. However, Prystaz et al. reported that while global inhibition of IL-6 significantly decreased the proportion of neutrophils in the FH, it also impaired the process of fracture healing [79]. Given the pleiotropic nature of IL-6, further studies are needed to explore the relationship between IL-6 and the N1/N2 phenotype in the context of bone regeneration. Apart from the interactions with inflammatory cells, neutrophils also interact with BMSCs [64]. N2 neutrophils have been shown to attract BMSCs by secreting SDF-1α [35], and conversely, BMSCs were able to convert N1 neutrophils into N2 neutrophils [80]. These findings contribute to the concept of a temporal shifting pattern from N1 at an early stage of healing to N2 later.

The conversion of neutrophil phenotypes is not an event that is only induced by cytokines or cells. Specific materials have the ability to support bone regeneration by the conversion of macrophages to the M2 phenotype [62,81–86]. Notably, materials were shown to support other tissue regeneration by converting neutrophil phenotype. Recent work by Li et al. showed that strontium encourages neutrophil phenotype conversion to N2, which stimulates M2 macrophage phenotype conversion, resulting in promoting angiogenesis and tissue regeneration [87]. This is a possible explanation for previous work suggesting that strontium can contribute to osteogenesis [68,88]. The frequent use of fixation materials in the field of bone regeneration makes these findings relevant and warrants further studies on the influence of specific materials on the behavior of neutrophil phenotypes.

Building upon the various scenarios of neutrophils during fracture healing as outlined above, the studies included in this review have detailed alterations in both the quantity and function of neutrophils [13,27–29,35–37,40–54]. Multiple studies have shown that changes in the neutrophil count can markedly influence the outcome of fracture healing, either positively or negatively [13,27,36,37,40–44,47,48,50]. This suggests a notable shift in the overall function of neutrophils in the context of bone healing. Intriguingly, in the context of diabetes, where the neutrophil count remains stable, alterations in neutrophil function impede fracture healing [45,46]. This reinforces the notion that distinct subpopulations of neutrophils may exist, each playing a distinct role during fracture healing. Examining whether N1/N2 phenotypes can offer an explanation becomes a compelling avenue of research. An increasing number of studies are now exploring the presence of N1/N2 neutrophils in various scenarios during bone healing *in vivo*, although it is still limited. The investigation into the existence and characteristics of N1/N2 neutrophils *in vivo*, especially in humans, holds great promise.

It is important to highlight that the majority of studies included in this systematic review primarily utilize murine experimental systems to investigate the role of neutrophils in bone regeneration. In mice, neutrophils make up 10–20 % of white blood cells [89], exhibit specific traits such as IL-10 secretion [90], and lack certain trafficking-related cytokines and receptors [91]. Despite these disparities from human neutrophils, under appropriate conditions, murine models can serve as potent and insightful experimental tools, offering valuable insights into human biology that may be challenging to attain through other means. Therefore, undoubtedly, integrating evidence from well-established murine models of neutrophil biology with *in vitro* testing of human neutrophils represents a solid approach for studying the relationship between neutrophils and bone regeneration. It is imperative to interpret conclusions drawn solely from mouse models with caution.

This systematic review has several limitations. First, we restricted our search to the PubMed database. While this is the most comprehensive database for clinical medicine, our search may have overlooked studies available in other databases. Second, the quality of the two included pre-clinical *in vitro* studies was not rigidly evaluated due to the lack of an appropriate assessment tool. Third, the number of included studies that investigated N1/N2 neutrophils in the field of bone regeneration was relatively low. Finally, it is worth noting that the generalizability of the conclusions drawn in each article may be limited, despite our evaluation, as indicated in Supplement 5. Our review emphasizes that the specific function attributable to either N1 or N2 neutrophils remains unclear. However, the early stage exploration of N1/N2 phenotypes in the field of bone research, where inflammation and regeneration intersect, is promising. New insights in this area could contribute to the field of tissue engineering in its broadest definition, as shown in Fig. 3.

5. Conclusions

In conclusion, this review identified several aspects and mechanisms

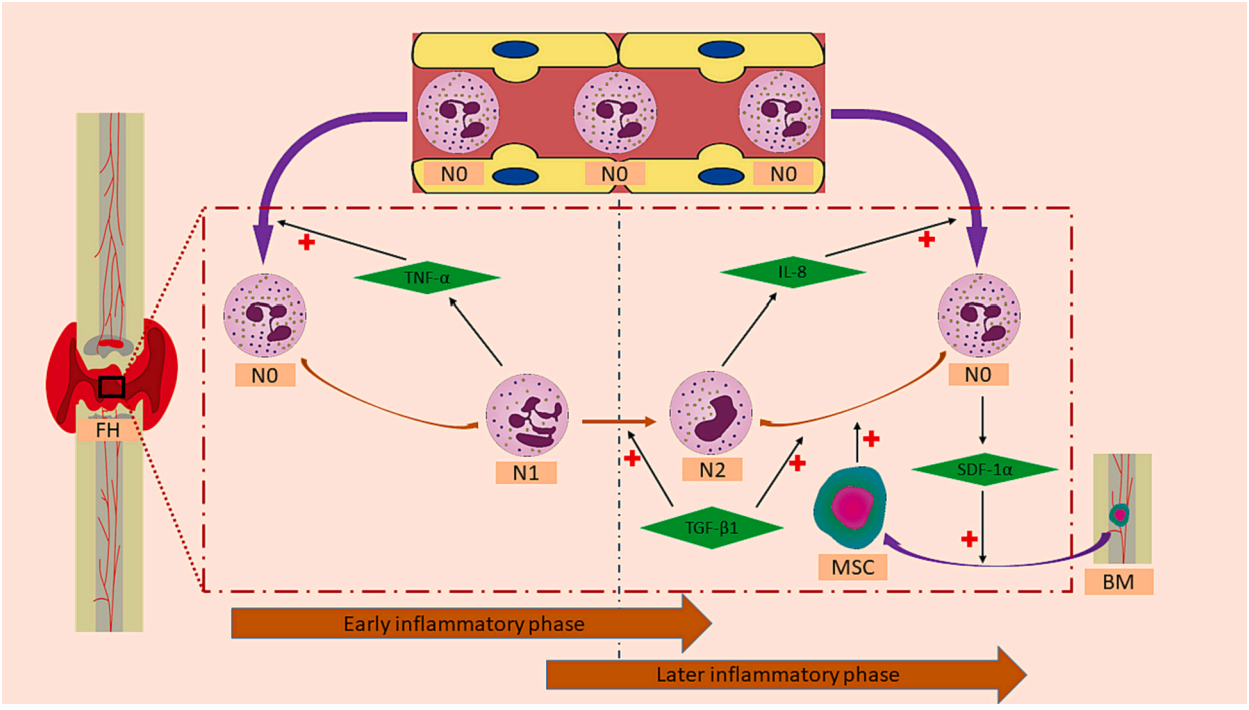

Fig. 3. Hypothesis of this systematic review. After a fracture occurs, during the early inflammatory phase, TNF-α participates in attracting circulating neutrophils to the FH. Through a mechanism that remains under investigation, N0 neutrophils can transition into N1 neutrophils, actively participating in the inflammatory response. In the later inflammatory phase, IL-8 is involved in attracting circulating neutrophils into the FH. TGF-β1 plays a crucial role in transforming both N0 and N1 phenotypes into N2 phenotypes. Additionally, by secreting SDF-1α, neutrophils facilitate the recruitment of MSCs into the fracture hematoma, thereby promoting the process of bone healing. Abbreviation: FH: Fracture Hematoma; BM: Bone Marrow; MSC: Mesenchymal stem cell; N0: Unstimulated Neutrophil Phenotype; N1: Neutrophil N1 Phenotype; N2: Neutrophil N2 Phenotype; TNF-α: Tumor Necrosis Factor-α; IL-8: Interleukin-8; TGF-β1: Transforming Growth Factor Beta 1; SDF-1α: Stromal Cell-Derived Factor-1α.

in the field of bone healing where neutrophils play an important role. The identification of N1 and N2 neutrophil phenotypes may provide new insights into the temporal changes that occur in the FH, with cytokine profiles and cell populations that change rapidly over time. The mechanisms described in this review support the idea that the neutrophil population in the FH is not homogenous, but consists of different phenotypes that change over time.

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CRediT authorship contribution statement

Fangzhou Lu: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Samai M.N.E. Verleg:** Writing – review & editing, Project administration, Investigation, Data curation. **Rald V.M. Groven:** Writing – review & editing, Methodology. **Martijn Poeze:** Writing – review & editing, Supervision. **Martijn van Griensven:** Writing – review & editing, Supervision. **Taco J. Blokhuis:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no conflict of interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2024.117021.

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