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INTEGRATED STUDY MASTER'S THESIS

Vascularisation of Composite Bone Scaffolds In Rodents with "Microfil" Perfusion. Systematic Review

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LIST OF ABBREVIATIONS

ALP	Alkaline Phosphatase
ASC	Adipose-Derived Stem Cell
BG	Bioactive Glass
BMD	Bone Mineral Density
BMP	Bone Morphogenetic Protein
BMSC	Bone Marrow-Derived Mesenchymal Stem Cell
BV/TV	Bone Volume Fraction
CS	Chitosan
CT	Computed Tomography
DMOG	Dimethyloxalylglycine
EMF	Electromagnetic Fields
EPC	Endothelial Progenitor Cells
FGF	Fibroblast Growth Factor
FTY	Bioactive Lipid FTY720
GF	Growth Factor
HA	Hydroxyapatite
HT	Hardystonite
HUVEC	Human Umbilical Vein Endothelial Cell
M	Mean Value
MS	Magnesium Silicate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBA	New Bone Area
NW	Nanowire
PHBHHx	Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)
PHMG	Combination of mesoporous BG and PHBHHx
PLA	Polylactic Acid
PLGA	Poly(lactide-co-glycolic acid)
PLLA	Poly-L-lactic acid

RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
SD	Sprague-Dawley
SEM	Scanning Electron Microscopy
Sr	Strontium
StD	Standard Deviation
TCP	Tricalcium Phosphate
TEM	Transmission Electron Microscopy
VA	Vascularised Area
VEGF	Vascular Endothelial Growth Factor
VN	Vessel Number
XRD	X-ray Diffraction
3D	Three-Dimensional

ABSTRACT

Introduction: Extensive research in oral and maxillofacial surgery aims to find an ideal alternative to autogenous bone for critical-size bony reconstructions. However, no such material has been found. Despite ongoing challenges, three-dimensional (3D) composite scaffolds show promise in improving vascularisation and bone regeneration by closely mimicking natural bone. A new investigation technique combining "Microfil" perfusion and micro-computed tomography (CT) allows efficient analysis of neovascularisation, bone regeneration, scaffold integration, and quantitative comparison between studies.

Purpose: This systematic review aims to investigate the effect of 3D composite scaffolds on new bone formation and vascularisation in critical-sized calvarial defects in rodents using "Microfil" perfusion and micro-CT.

Method: A comprehensive electronic search was conducted according to the PRISMA guidelines in PubMed and Medline from January 2013 to October 2023 limited to English language publications with available full texts. *In vivo* studies about "vascularisation bone scaffolds" using scaffolds made from a combination of inorganic and organic synthetic materials and analysing the neovascularisation and bone regeneration with "Microfil" and micro-CT techniques were investigated.

Results: The full text of 48 studies was assessed for eligibility, with 8 studies meeting the inclusion criteria. Among these, scaffolds with larger pore sizes (≥400 μm) exhibited a trend towards improved vascularisation and bone regeneration outcomes ($p<0.05$ for bone volume fraction (BV/TV), $p=0.053$ for vascularised area (VA)). Additionally, composite scaffolds showed significantly higher new bone area (NBA) than non-composite scaffolds ($p<0.05$). Moreover, modified scaffolds incorporating angiogenic and/or osteogenic factors excelled pure scaffolds in vascularisation and bone regeneration ($p<0.01$ for NBA, $p<0.05$ for VA and BV/TV, $p=0.051$ for vessel number (VN)). Combining two modifying factors generated even better results ($p<0.01$ for VN and NBA, $p<0.05$ for BV/TV, $p=0.071$ for VA). Nevertheless, three included studies showed a high risk of bias in at least one category, and all included studies presented missing information in half of the assessed items.

Conclusion: Enhancing vascularisation and bone regeneration in critical-sized calvarial defects using 3D composite scaffolds may benefit from modifications with angiogenic and/or osteogenic factors, especially when delivered together and with larger pore sizes. A properly designed scaffold structure could potentially erase the need for adding angiogenic and/or osteogenic factors. Future studies with larger sample sizes and similar study designs should investigate the optimal morphology as well as osseo- and angioinductive properties of these scaffolds.

Keywords: Composite scaffolds; Vascularisation; Microfil; Micro-CT; Growth factors; Bone tissue engineering.

1 INTRODUCTION

Over the years, bone graft procedures have become increasingly popular. Worldwide, more than four million surgeries are performed every year using bone grafts and bone substitutes (1). Bone is the second most transplanted tissue after blood transfusions (2). Especially in oral and maxillofacial surgery, the interest in bony reconstructions has grown drastically in recent years as dental implant popularity has increased (3). In 2020, the dental bone graft substitute market size was estimated at USD 450 million with an expected annual growth of 7.9% reaching USD 659 million by 2025 (4). Bone grafts or bone substitute materials are regularly necessary to regenerate critical-size bone defects despite the bone's ability to self-heal (5–8). Various sizes of dental bone defects can be caused by trauma, periodontal disease, and dental extractions following caries, apical periodontitis, or other pathologies (9).

Even though various bone substitute materials have been tested throughout the years, an ideal bone substitute material is still missing. Available biomaterials cannot withstand various loads while keeping an appropriate porosity to encourage cell growth and vascularisation of the regenerating bone (5). Therefore, autogenous bone grafts are still the gold standard for reconstructing large-size defects (5,10). Compared to other bone substitutes, autografts can predictably increase bone volume and quality, improving possibilities for implant placement and long-term success (10). However, limited availability, donor site complications (e.g. infections, injury, movement impairments), and morphological limitations may restrict the clinical application (11). These limitations cause a need for alternative ways of bone regeneration, opening possibilities in the field of bone tissue engineering. Bone tissue engineering creates scaffolds that function as structural conduits for bone growth and mechanical support in load-bearing areas ensuring cell attachment, proliferation, and osteogenic function. Such properties are needed to secure structural integrity till new bone takes over (5).

The field of bone tissue engineering has been exploring ways of transforming advanced biomaterials into porous, load-resisting three-dimensional (3D) scaffolds. Various synthetic materials, including ceramics, composites, polymers, and metals, have been developed as a potential bone substitute alternative (5,12). With their unlimited availability, variation in size and shape, as well as by modifying their biological, chemical, and physical features, bone scaffolds have the chance to substitute auto- and allografts. Particularly, composite scaffolds that incorporate organic and inorganic materials can closely mimic natural bone (13). The internal structure and, with it, the biological and mechanical features of those scaffolds can be modified in detail and enhanced by bone-specific growth factors (GFs) and cells to improve tissue regeneration (5,14).

By employing new investigation techniques, particularly “Microfil” perfusion and micro-computed tomography (CT) in combination, it is possible to investigate bone vascularity, scaffold vascularisation, and overall bone quality with the help of high-resolution 3D images (15). Combining

"Microfil" perfusion and micro-CT, sometimes referred to as a multimodal imaging approach (16), aids in overcoming weaknesses of previous methods allowing clear visualisation of blood vessels and precise localisation of vascularised area (VA) within the bone. This novel technique facilitates the analysis of correlations among vascular patterns, bone regeneration, and scaffold integration to help improve scaffold morphology and design. As a non-destructive technique, it enables subsequent analysis on the same specimen, enhancing efficiency (15). Moreover, the quantitative analysis facilitated by this investigative technique enables the measurement of parameters such as vessel number (VN), VA, bone volume fraction (BV/TV), bone mineral density (BMD), and new bone area (NBA), improving the comparability between studies.

Even though technologies are continuously progressing, it is still challenging to produce grafts that have both natural bone's biological and mechanical properties, ensuring successful vascularised bone formation (14).

1.1 RESEARCH AIM

This systematic review aims to investigate the effect of 3D composite scaffolds on new bone formation and vascularisation in critical-sized calvarial defects in rodents using "Microfil" perfusion and micro-CT.

1.2 RESEARCH OBJECTIVES

The following research objectives were developed:

To determine the importance of scaffold morphology, particularly of pore sizes, on neovascularisation and new bone formation outcomes.

To investigate whether composite materials, when compared to non-composite materials, influence neovascularisation and new bone formation outcomes.

To evaluate the impact of scaffold modifications, both single- and dual-factor enhancements, on neovascularisation and new bone formation outcomes.

2 MATERIALS AND METHODS

2.1 ELIGIBILITY CRITERIA

The applied inclusion criteria of this study were as follows:

Studies concerning "vascularisation bone scaffolds"

Studies performed *in vivo*

Studies performing "Microfil" perfusion

Studies implanting scaffolds into rodents

Studies using 3D composite scaffolds composed of a combination of inorganic and organic synthetic materials

Studies performing surgeries in the skull region

The applied exclusion criteria for this study were as follows:

Studies that were systematic reviews or reviews

Studies that were performed only *in vitro*

Studies that did not perform “Microfil” perfusion

Studies using scaffolds on other animals than previously explained in inclusion criteria

Studies using other scaffolds than previously explained in inclusion criteria

2.2 INFORMATION SOURCES AND SEARCH

An electronic search was conducted in PubMed and Medline databases from January 2013 to October 2023, limited to English language publications with available full texts. Published papers on the selected topic were found using the following keywords: (((Vascularisation bone scaffolds) NOT (systematic reviews) NOT (reviews) AND (*in vivo*) AND (rodents) OR (microfil))) OR ((Vascularisation bone scaffolds) NOT (systematic reviews) NOT (reviews) NOT (*in vitro*) AND ((rodents) OR (microfil)))). Figure 1 shows the flow chart diagram of the present study selection according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analysis) guidelines.

The main objective was to answer the following “PICOT” (P=Patient/Problem/Population; I=Intervention; C=Comparison; O=Outcome; T=Time) question: In rodents (P), what effect does the critical-sized calvarial defect repair with 3D composite scaffolds (I) have on the vascularisation of the defect area (O) comparing different composite materials and scaffold designs (C) within 4-12 weeks after surgery (T).

2.3 STUDY SELECTION

Study selection and data extraction were performed by two independent reviewers, and a third independent expert resolved disagreements. The primary selection of studies was based on the inclusion criteria. Systematic reviews, reviews, and *in vitro* studies were not considered. During the search, it was detected that the “Microfil” perfusion was rarely mentioned in the abstracts or keywords. That is why it was decided to search the full texts for the word "Microfil" at this stage. The remaining articles were assessed for eligibility based on the inclusion criteria. Full texts of eligible studies were completely reviewed.

2.4 POPULATION SELECTION

Studies of rodents in which bone scaffolds were transplanted in critical-sized calvarial bony defects and evaluated for vascularisation via “Microfil” perfusion.

2.5 TYPE OF INTERVENTIONS

Studies performing *in vivo* surgery on rodents that created a critical-size bone defect in the skull region and repaired it with composite scaffolds were included and analysed with "Microfil" perfusion.

2.6 TYPE OF OUTCOME MEASUREMENT

Research that reported the effect of the surgical intervention on the vascularisation and bone regeneration of the defect area (VN, VA, BV/TV, BMD, and NBA) regarding scaffold material, additives, design, geometry, and time of "Microfil" perfusion was included.

2.7 DATA ITEMS

Of all included articles, the following information was extracted: **1) Animals:** age, gender, and strain of rats used for *in vivo* investigations; **2) Sample size, study groups, and defect features:** type and count of the samples used for "Microfil" perfusion, micro-CT, and histology, number and characteristics of study groups, and size of the created defect; **3) Analysis methods:** methods used to evaluate the vascularisation and bone regeneration of the defect site (micro-CT, "Microfil" perfusion, histology); **4) Timing of "Microfil" perfusion, micro-CT, and histology:** the number of weeks post-surgery after which "Microfil" perfusion, micro-CT, and histology was performed; **5) Vascularisation analysis:** data regarding VN and VA in the critical-sized bony defect; **6) New bone formation analysis:** data regarding NBA, BV/TV, and BMD in the critical-sized bony defect; **7) Scaffold design:** measurements and geometry of scaffolds, as well as size and arrangement of pore structure; **8) Scaffold composition**: types of materials used to fabricate the employed scaffolds; **9) Scaffold modification:** GFs or other modifications added to scaffolds after their initial production for *in vivo* experiments.

2.8 STATISTICS

Statistical analyses were performed to evaluate the influence of various scaffold characteristics (pore size, scaffold material, single-factor modification, and dual-factor modification) on vascularisation and bone regeneration outcomes. The effect of pore size was analysed by grouping the data in scaffolds with big pores (≥400 μm) and small pores (<400 μm). The impact of scaffold material was evaluated by sorting the data according to pure composite scaffolds and simple non-composite scaffolds, both without modifications. The data was further sorted according to scaffolds with and without modifications for analysing the influence of scaffold modifications. Control groups were not included to facilitate the direct comparison between pure scaffolds and modified ones. To make the studies statistically comparable estimates of the vascularisation and bone regeneration results were taken from graphics when they did not specify the numbers in their written text.

After performing descriptive analysis, tests of normality, and Pearson correlation coefficients for the dependent variables (VN, VA, BV/TV, BMD, NBA, and porosity), independent samples tests (t-tests) were calculated. Two-sided p-values were evaluated for statistical hypothesis testing when not explicitly specified otherwise.

2.8.1 SCAFFOLD DESIGN

To assess if there is a difference between composite scaffolds with big pores and composite scaffolds with small pores, independent t-tests were performed. The null hypothesis H0: "The pore size of a composite scaffold does not influence vascularisation and bone regeneration" was tested. Alternative hypotheses were formulated: "A big pore size (≥400 μm) of a composite scaffold improves vascularisation and bone regeneration when compared to a small pore size (<400 μm)" (H1, one-sided test), and "The pore size of a composite scaffold influences vascularisation and bone regeneration" (H2, two-sided test).

2.8.2 SCAFFOLD COMPOSITION

Independent t-tests were performed to compare pure composite scaffolds and simple non-composite scaffolds, both without modifications, to investigate whether there is a difference in vascularisation and bone regeneration. The null hypothesis H0: "The use of composite scaffold material does not influence vascularisation and bone regeneration" was tested. An alternative hypothesis was formulated: "The use of composite scaffold material influences vascularisation and bone regeneration" (H1, two-sided test).

2.8.3 SCAFFOLD MODIFICATION

Independent t-tests were conducted to assess the difference between scaffolds with modifications and those without, and between scaffolds enhanced by one angiogenic and/or osteogenic factor and those with two. The null hypotheses H0 (modification yes/no): "Composite scaffold modification does not influence vascularisation and bone regeneration", and H0 (number of modifications): "The number of osteogenic factors used to enhance a composite scaffold does not influence vascularisation and bone regeneration" were tested. The following alternative hypotheses were formulated: "Composite scaffold modification improves vascularisation and bone regeneration" (H1 (modification yes/no), one-sided test), "Composite scaffold modification influences vascularisation and bone regeneration" (H2 (modification yes/no), two-sided test), "Dual-factor modification used to enhance composite scaffolds improves the vascularisation and bone regeneration when compared to a single-factor modification" (H1 (number of modifications), one-sided test), and "The number of osteogenic factors used to enhance a composite scaffold influences vascularisation and bone regeneration" (H2 (number of modifications), two-sided test).

2.9 REPORTING BIAS ASSESSMENT

SYRCLE´s risk of bias tool (17) was used to evaluate the risk of bias in each of the included animal studies. This approach is consistent with previous studies (18,19).

Since it was impossible to acknowledge each factor´s weight for the overall assessment, an overall risk of bias is not presented (17).

3 RESULTS

3.1 RESULTS: STUDY SELECTION

A total of 1165 articles were identified from PubMed and Medline searches. After removing duplicates, 595 articles were sought for retrieval when forty-seven records could not be retrieved. Five hundred studies were excluded for not using “Microfil” perfusion. Forty-eight articles were included as relevant for this systematic review. After assessing full texts for eligibility, forty studies were excluded due to the following reasons: first, preparation of surgical defect site in a region other than skull, i.e., improper defect site; second, fabricating scaffolds with materials other than a combination of inorganic and organic synthetic composites, i.e., improper scaffold material; third, those performing surgery on other animals than rodents, i.e., improper animal. Finally, eight studies were considered for comprehensive analyses, as illustrated in the PRISMA flow diagram (Fig. 1).

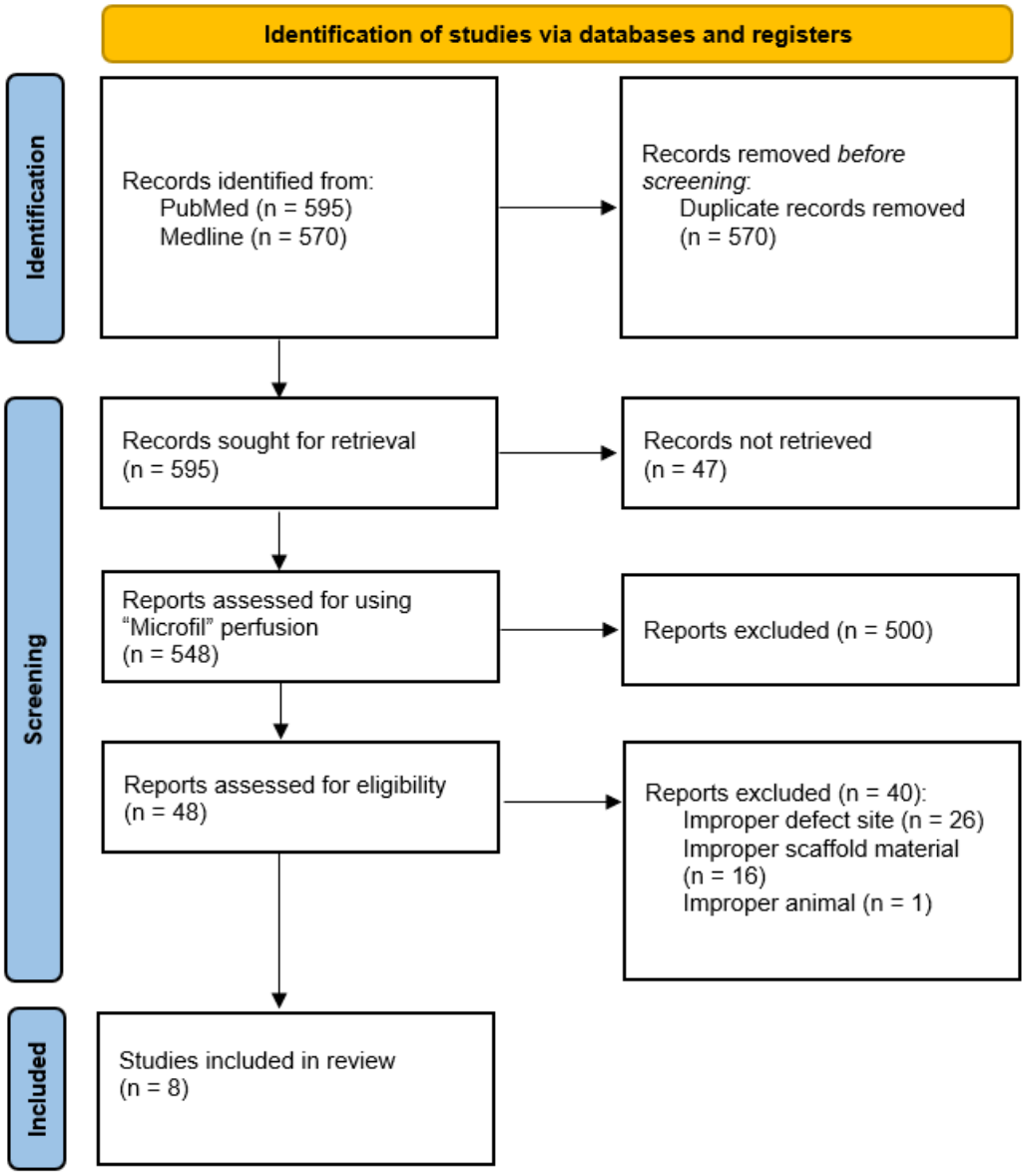

Fig. 1. PRISMA Flow Diagram.

3.2 RESULTS: STUDY CHARACTERISTICS

The study characteristics of all included studies were obtained and summarised in Table 1.

3.2.1 ANIMALS

According to the inclusion criteria, rodents were included in the search. After excluding articles due to "improper defect site", "improper scaffold material", and not full-text availability, the remaining eight articles had all performed the surgeries on rodents, more specifically rats, only (Table 1). Seven of these studies (20–26) utilized male Sprague-Dawley (SD) rats. However, there was an exception: Kuttappan S. et al.'s study (27), which performed their experiments on 4-5-month-old Wistar male rats. The age of the SD rats varied across the other studies, ranging from 8 weeks (20,21) to 12-13 weeks (22,24–26), with one study simply indicating that the rats were mature (23).

3.2.2 SAMPLE SIZE, STUDY GROUPS, AND DEFECT FEATURES

Sample size, study groups, and defect features varied across the included studies (Table 1). The sample size used for animal experiments varied between 12 (22,25) and 126 (26) animals. The study by Kuttappan S. et al. (27) was the only study not reporting a sample size. Furthermore, the sample sizes varied depending on the performed analysis: the study by Li S. et al. (21) divided the rats into four groups with six rats per group, however, only three rats per group were used for "Microfil" and micro-CT analysis. Tu C. et al. (26) used only six out of 24 rats per group for the "Microfil" analysis at 8 weeks, and six rats of each group for micro-CT analysis at 4 and 12 weeks. The number of study groups was as follows: 2 groups (25), 3 groups (20,24), 4 groups (21–23), 5 groups (26), and 6 groups (27). Four studies included control groups (21,24,26,27) that did not receive a defect repair with a scaffold. Six studies created two defects with a diameter of 5 mm per rat (20–25) and two studies created one defect per rat with a diameter of 8 mm (27) and 6 mm (26).

3.2.3 ANALYSIS METHODS

All included studies performed "Microfil" perfusion and histological analysis for their *in vivo* experiments (Table 1). Additionally, studies performed fluorescence labelling (21–24), histomorphometric analysis (22,27), immunohistochemistry (23), immunofluorescence assay (21), and biomechanical analysis (26).

3.2.4 TIMING OF "MICROFIL" PERFUSION, MICRO-CT, AND HISTOLOGY

The included studies performed "Microfil" perfusion and micro-CT at different time points (Table 1). Four studies reported "Microfil" perfusion at 8 weeks after surgical implantation of the scaffolds (21–24), others at 4 weeks (27), 6 weeks (26), 12 weeks (20), and 4 and 8 weeks (25). All studies performed micro-CT analysis at the same time points as stated for the "Microfil" perfusion,

except for the study by Tu C. et al. (26), which performed micro-CT analysis for bone regeneration assessment at weeks 4 and 12.

3.2.5 VASCULARISATION ANALYSIS

All studies, except for the study by Min Z. et al. (24), reported the VA of defect sites after scaffold implantation. However, only four studies (20,23,25,26) recorded the VN (Table 1). The study by Wang Y. et al. (25) was the only study reporting vascularisation results at two-time points (4 and 8 weeks). The study by Kuttappan S. et al. (27) was the only study to report the VA in fold increase, other studies reported the data in per cent.

3.2.6 NEW BONE FORMATION ANALYSIS

The included studies analysed the new bone formation by reporting different parameters (Table 1). Overall, six studies (20–23,26,27) reported results for NBA, five studies (20,21,23,25,26) for BV/TV, and three studies (23,25,26) for BMD. Three studies (25–27) reported their results for two time points (4 and 12 weeks (26,27); 4 and 8 weeks (25)).

Table 1. Data Items.

Author(s) and year	1) Animals	2.1) Sample Size	2.2) Study Groups	2.3) Defect Features	3) Analysis Methods	4.1) "Microfil" and Micro-CT	4.3) Histology
Qi X. et al. 2017	Male SD rats, mature	24 animals (n=6 per group)	4 (Group A: PHMG; Group B: PHMB; Group C: PHMD; Group D: PHMBD)	Ø 5 mm (2 defects per rat)	In vivo: Microfil, Micro-CT, histology, fluorescence labeling, and immunohistochemical analysis; in vitro: RT-qPCR analysis, western blotting	at 8 weeks	at 2, 4, and 6 weeks
Min Z. et al. 2015	Male SD rats, 12-week-old	12 animals (n=6 for G1, G2)	3 (Group A: PHMG; Group B: PHMD; Group C: control)	Ø 5 mm (2 defects per rat)	In vivo: Microfil, Micro-CT, histology, sequential fluorescence labelling; in vitro: cell attachment, cytotoxicity, ALP activity, RT-qPCR analysis	at 8 weeks	at 2, 4, and 6 weeks
Kuttappan S. et al. 2018	Male Wistar rats, 4-5-month-old	n=?	6 (Group A: Sc; Group B: Sc/B; Group C: Sc/V; Group D: Sc/F; Group E: Sc/B/V; Group F: Sc/B/F)	Ø 8 mm (1 defect per rat; thickness 1.5 mm)	In vivo: Microfil, Micro-CT, histology, and histomorphometry; in vitro/in vivo: growth factor release, cytocompatibility, osteogenic differentiation, endothelial functionality	at 4 weeks	at 4 and 12 weeks (12 w. used for statistics)
Sun T.W. et al. 2017	Male SD rats, 8-week-old	24 animals (n=8 per group)	3 (Group A: CS; Group B: HANWs/CS; Group C: HANW@MS/CS)	Ø 5 mm (2 defects per rat)	In vivo: Microfil, MicroCT, histology; in vitro: SEM, TEM, and XRD, drug loading analysis, analysis of cytoskeleton staining and SEM micrographs, RT-qPCR analysis	at 12 weeks	at 12 weeks
Wang Y. et al. 2023	Male SD rats, 12-week-old	12 animals (n=6 at 4 and 8 weeks)	2 (Group A: PLA/HA; Group B: VEGF+PLA/HA)	Ø 5 mm (2 defects per rat)	In vivo: Microfil, Micro-CT, histology; in vitro: immunofluorescence staining, scanning electron microscopy, energy spectrum analysis	at 4 and 8 weeks (8 w. used for statistics)	at 4 and 8 weeks
Li S. et al. 2019	Male SD rats, 8-week-old	24 animals (n=6 (3 for Microfil) per group)	4 (Group A: control; Group B: PLGA; Group C: MBG-PLGA; Group D: FTY/MBG-PLGA)	Ø 5 mm (2 defects per rat)	In vivo: Microfil, Micro-CT, histology, sequential fluorescence labeling, immunofluorescence assay of CD31 and Emcn; in vitro: angiogenesis assay of HUVECs	at 8 weeks	at 4 and 6 weeks
Tu C. et al. 2020	Male SD rats, 12-13-week-old	126 animals (n=24 (6 for Microfil) per group)	5 (Group A: control; Group B: PLA-HA; Group C: PLA-HA/EMF; Group D: PLA-HA/BMSCs; Group E: PLA-HA/BMSCs/EMF)	Ø 6 mm (1 defect per rat)	In vivo: Microfil, Micro-CT, histology, biomechanical analysis; in vitro: scanning electron microscopy, CCK-8 assay and a LIVE/DEAD kit	"Microfil" at 6 weeks, micro-CT at 4 and 12 w.	at 4 and 12 weeks
Wang G. et al. 2017	Male SD rats, 12-week-old	16 animals (n=4 per group)	4 (Group A: TCP/HA; Group B: Sr-HT-Gahnite; Group C: TCP/HA+ASCs; Group D: Sr-HT-Gahnite+ASCs)	Ø 5 mm (2 defects per rat)	In vivo: Microfil, fluorochrome labeling histomorphometric analysis, histology; in vitro: ASCs culture, semi-quantitative study (ALP activity), Alizarin Red S staining, Ion concentrations (ICP-AES), expression of angiogenic genes, HUVECs culture (MTT assay, transwell assay, mRNA expression levels)	at 8 weeks	at 4, 6, and 8 weeks

Table 1. Data Items (continued).

Author(s) and year	5) Vascularisation Analysis: VN, VA	6) New Bone Formation Analysis: NBA, BV/TV, BMD
Qi X. et al. 2017	VN: PHMG ≈ 3; PHMB ≈ 30; PHMD ≈ 40; PHMBD ≈ 90; VA in %: PHMBD ≈ 86.09; PHMD ≈ 36.11; PHMB ≈ 21.65; PHMG ≈ 1.27	NBA (in %): PHMBD ≈ 89.5; PHMG ≈ 4.50; PHMB ≈ 26.17; PHMD ≈ 14.00; BV/TV (in %): PHMG ≈ 3; PHMB ≈ 35; PHMD ≈ 15; PHMBD ≈ 53; BMD (in g/cm^3): PHMG <0.1; PHMB ≈ 0.5; PHMD≈ 0.3; PHMBD 0.88
Min Z. et al. 2015	not specified	not specified
Kuttappan S. et al. 2018	VN: not specified; VA in fold increase (in regards to control): Sc <1; ScB ≈ 2; ScV >3<4; ScF ≈ 2; ScBV ≈ 4; ScBF >4<5	NBA (in %): Control ≈ 0 at 4w., 1,5 at 12 w.; Sc ≈ 3 at 4w., 37 at 12w.; ScB ≈ 48 at 4w., 80 at 12w.; ScV ≈ 4 at 4w., 45 at 12w.; ScF ≈ 3 at 4w., 51 at 12w.; ScBV ≈ 52 at 4w., 87 at 12w.; ScBF ≈ 56 at 4 w., 92 at 12 w.; BV/TV and BMD not specified
Sun T.W. et al. 2017	VN: CS ≈ 13; HANWs/CS ≈ 29; HANW@MS/CS ≈ 50; VA in %: CS 5.38 %; HANWs/CS ≈ 9.66 %; HANW@MS/CS ≈ 13.26 %	NBA (in %): CS ≈ 3.15; HANWs/CS ≈ 22.99; HANW@MS/CS ≈ 39.41; BV/TV (in %): CS ≈ 4.92; HANWs/CS ≈ 25.06; HANW@MS/CS ≈ 40.15; BMD not specified
Wang Y. et al. 2023	VN: PLA/HA ≈ 16 at 4w., 17 at 8w.; PLA/HA+VEGF≈ 24 at 4w., 25 at 8w; VA in %: PLA/HA ≈ 25 at 4w., 35 at 8w.; PLA/HA+VEGF ≈ 38 at 4w., 64 at 8w.	NBA not specified; BV/TV (in %): PLA/HA ≈ 0.03 at 4w., 0.1 at 8w.; PLA/HA+VEGF ≈ 0.03 at 4w., 0.13 at 8w.; BMD (in g/cm^3): PLA/HA ≈ 1.03 at 4w., 1.05 at 8w.; PLA/HA+VEGF ≈ 1.05 at 4w., 1.10 at 8w.
Li S. et al. 2019	VN: not specified; VA in %: Control ≈ 1.5; PLGA ≈ 4.10; MBG-PLGA ≈ 10.25; FTY/MBG-PLGA ≈ 21.07	NBA (in %): Control ≈ 1; PLGA ≈ 2.83; MBG-PLGA ≈ 7.81; FTY/MBG-PLGA ≈ 16.6; BV/TV (in %): Control ≈ 2.5; PLGA ≈ 4; MBG-PLGA ≈ 9.15; FTY/MBG-PLGA ≈ 17.47; BMD not specified
Tu C. et al. 2020	VN: Control ≈ 5; Scaffold ≈ 20; Scaffold/EMF ≈ 35; Scaffold/BMSCs ≈ 40; Scaffold/BMSCs/EMF ≈ 80; VA in %: Control ≈ 2.5; Scaffold ≈ 10; Scaffold/EMF ≈ 21; Scaffold/BMSCs ≈ 23; Scaffold/BMSCs/EMF ≈ 35	NBA (in %): Control ≈ 3 at 4w., 5 at 12 w.; Scaffold ≈ 10 at 4w., 21 at 12 w.; Scaffold/EMF ≈ 20 at 4 w., 40 at 12 w.; Scaffold/BMSCs ≈ 21 at 4 w., 41 at 12 w.; Scaffold/BMSCs/EMF ≈ 30 at 4 w., 58 at 12 w.; BV/TV (in %): Control ≈ 2 at 4w., 5 at 12w.; Scaffold ≈ 9 at 4w., 21 at 12w.; Scaffold/EMF ≈ 20 at 4w., 43 at 12w.; Scaffold/BMSCs ≈ 23 at 4w., 48 at 12w.; Scaffold/BMSCs/EMF ≈ 34 at 4w., 73 at 12w.; BMD (in mg/cm^3): Control ≈ 20 at 4w., 50 at 12w.; Scaffold ≈ 70 at 4w., 170 at 12 w.; Scaffold/EMF ≈ 160 at 4w, 320 at 12 w.; Scaffold/BMSCs ≈ 180 at 4w., 330 at 12 w.; Scaffold/BMSCs/EMF ≈ 260 at 4w., 430 at 12w.
Wang G. et al. 2017	VN: not specified; VA in %: TCP/HA ≈ 1.9; TCP/HA/ASCs ≈ 2.8; Sr-HT-gahnite ≈ 3.0; Sr-HT-gahnite/ASCs ≈ 6.7	NBA (in %): TCP/HA ≈ 0.5; TCP/HA/ASCs ≈ 6.6; Sr-HT-gahnite ≈ 5.8; Sr-HT-gahnite/ASCs ≈ 14.5; BV/TV and BMD not specified

Table 1. Data Items (continued).

Author(s) and year	7) Scaffolds Design: measures, geometry, pore size, porosity, compressive strength	8) Scaffold Composition	9) Scaffold Modification
Qi X. et al. 2017	5x5 mm; macropores 450–900 µm, micropores 20–50 µm (on frame walls); porosity 80%	mesoporous BG-PHBHHx (PHMG)	DMOG, BMP-2
Min Z. et al. 2015	6x3 mm; cylindrical shape; well-defined square pore structure (ordered, uniform mesoporous), 700 µm between strands	mesoporous BG-PHBHHx (PHMG)	DMOG
Kuttappan S. et al. 2018	8x1.5 mm; pore size 50–350 µm; porosity 58.8% ± 7.3%; overall compressive strength of 28 ± 3.5 MPa	nanoHA-silica gelatinous matirx-PLLA (Sc)	BMP2, FGF2, VEGF
Sun T.W. et al. 2017	5x2 mm; pore sizes 200-300 µm; compressive strength: HANW@MS/CS 6.18, HANWs/CS 7.84, pure CS 5.38 kPa	CS/ CS-HANWs/ CS-HANW@MS	/
Wang Y. et al. 2023	5 mm; circular membrane, irregular spun woven structures, uniform spinning morphology; homogenous pores; porosity 63%	PLA-HA	VEGF
Li S. et al. 2019	12x2 mm; pore size 257 ± 50 µm (PLGA), 252 ± 45 µm (MBG-PLGA); porosity 81% (PLGA), 82% (MBG-PLGA)	PLGA/ PLGA-MBG (mesoporous BG)	FTY
Tu C. et al. 2020	6x0.6 mm; cylindrical shape; pore diameter 1000 µm; porosity 70 ± 2.23%; compression strength 31.18 ± 4.86 MPa	PLA-HA	EMF, BMSCs
Wang G. et al. 2017	Defect 5 mm; pore size 400-700 µm (TCP/HA), 500 µm (Sr-HT-Gahnite); porosity 91% (TCP/HA), 85% (Sr–HT–Gahnite)	TCP-HA/ Sr-HT-Gahnite	ASCs

3.3 RESULTS: STATISTICS

All eight included studies were part of the statistical analysis. However, one study (24) did not quantify their vascularisation and bone regeneration results, while another study (27) expressed the results for VA solely in fold increase relative to their control group. Therefore, the mentioned results had to be excluded from the corresponding parts of the analysis.

Descriptive statistics were performed on the data set. The sample size was between n=10 and n=24 for the dependent variables listed below (Table 2). The following mean values (M) and standard deviations (StD) were found: VN (M=36.31, StD=25.03), VA (M=20.56, StD=22.09), BV/TV (M=24.50, StD=22.00), BMD (M=0.52, StD=0.36), NBA (M=33.60, StD=29.53), and porosity (M=72.82, StD=11.33).

Table 2. Descriptive Statistics.

	N	Minimum	Maximum	Mean	Std. Deviation
VN	13	3.00	90.00	36.3077	25.03459
VA	20	1.27	86.09	20.5620	22.09440
BV/TV	16	0.10	73.00	24.4988	21.99570
BMD	10	0.10	1.10	0.5180	0.36190
NBA	24	0.50	92.00	33.5983	29.53030
Porosity	23	58.80	91.00	72.8174	11.32991

A Shapiro-Wilk test was performed on a small sample of data (Table 3). The W statistics ranged from 0.833 to 0.984, and p-values ranged from 0.064 to 0.978, except for the dependent variable "Porosity" (W statistic of 0.665 and a p-value of <0.001) indicating that the data were not normally distributed. The p-values of all other dependent variables were greater than the significance level of 0.05, which does not allow us to reject the null hypothesis that the data is normally distributed. These results are supported by the Kolmogorov-Smirnov test (Table 3).

Table 3. Tests of Normality.

	Kolmogorov-Smirnov			Shapiro-Wilk		
	Statistic	**df**	**Sig.**	**Statistic**	**df**	**Sig.**
VN	0.281	8	0.063	0.911	8	0.362
VA	0.270	8	0.089	0.833	8	0.064
BV/TV	0.126	8	0.200*	0.984	8	0.978
BMD	0.206	8	0.200*	0.895	8	0.259
NBA	0.188	8	0.200*	0.936	8	0.572
Porosity	0.325	8	0.013	0.665	8	<0.001

*. This is a lower bound of the true significance.

The Pearson correlation coefficients were calculated between all dependent variables to assess their linear relationship. The results are summarised in Table 4.

A strong negative correlation between porosity and NBA was observed in the studied population, characterised by a Pearson correlation coefficient of r=-0.726 (p<0.001, n=21). Additionally, there are strong positive correlations between the following pairs of variables: VN and BV/TV (r=0.835, p<0.001, n=13), VN and NBA (r=0.926, p<0.001, n=11), VA and BMD (r=0.771, p=0.009, n=10), VA and NBA (r=0.868, p<0.001, n=18), BV/TV and NBA (r=0.883, p<0.001, n=14), and BMD and NBA (r=0.875, p=0.004, n=8). All those correlations were statistically significant at the $\alpha = 0.01$ level, indicating that the relationships between these variables are unlikely to be due to chance.

A moderate negative correlation was observed between porosity and VA, with a Pearson correlation coefficient of r=-0.491 (p=0.046, n=17). Similarly, a moderate positive correlation was observed between VN and VA, with a Pearson correlation coefficient of r=0.630 (p=0.021, n=13).

Both correlations were statistically significant at the $\alpha = 0.05$ level, indicating that the relationship between porosity and VA, and VN and VA are unlikely to be due to chance.

Table 4. Pearson Correlation.

		VN	VA	BV/TV	BMD	NBA	Porosity
VN	Pearson Correlation	1	0.630*	0.835**	0.194	0.926**	0.208
	Sig. (2-tailed)		0.021	<0.001	0.592	<0.001	0.565
	N	13	13	13	10	11	10
VA	Pearson Correlation	0.630*	1	0.293	0.771**	0.868**	-0.491*
	Sig. (2-tailed)	0.021		0.271	0.009	<0.001	0.046
	N	13	20	16	10	18	17
BV/TV	Pearson Correlation	0.835**	0.293	1	-0.209	0.883**	-0.031
	Sig. (2-tailed)	<0.001	0.271		0.563	<0.001	0.920
	N	13	16	16	10	14	13
BMD	Pearson Correlation	0.194	0.771**	-0.209	1	0.875**	-0.490
	Sig. (2-tailed)	0.592	0.009	0.563		0.004	0.150
	N	10	10	10	10	8	10
NBA	Pearson Correlation	0.926**	0.868**	0.883**	0.875**	1	-0.726**
	Sig. (2-tailed)	<0.001	<0.001	<0.001	0.004		<0.001
	N	11	18	14	8	24	21
Porosity	Pearson Correlation	0.208	-0.491*	-0.031	-0.490	-0.726**	1
	Sig. (2-tailed)	0.565	0.046	0.920	0.150	<0.001	
	N	10	17	13	10	21	23

***. Correlation is significant at the 0.05 level (2-tailed).**

****. Correlation is significant at the 0.01 level (2-tailed).**

3.3.1 SCAFFOLD DESIGN

The analysed studies presented various scaffold designs (Table 1). Included scaffolds were between 5 and 12 mm in length and 0.6 to 5 mm in height (20–24,26,27), except for one membrane with a diameter of 5 mm and no reported height (25). The scaffolds' designs ranged from disordered, irregular (23,25) to uniform, well-defined (24) structures. The scaffold porosity ranged from 58.8% (27) to 91% (22).

The analysed studies presented various pore sizes ranging from 20 (23) to 1000 μm (26). Only one study created a scaffold with both macropores in the centre and micropores on the frame walls (23). The studies were grouped according to their pore size into four groups: group 1 (20,21,27) “small pores” (pores <400 μm), group 2 (22,24,26) “big pores” (pores ≥400 μm), group 3 “others” (23) (micro- and macropores), group 4 (25) “not mentioned” (pore size not mentioned).

Independent samples t-tests comparing big (≥400 μm) and small (<400 μm) pores were performed (Table 5) demonstrating the following results: VN results for three scaffolds with small pores and four scaffolds with big pores, VA results for six scaffolds with small pores and four scaffolds with big pores, BV/TV results for six scaffolds with small pores and four scaffolds with big pores, BMD results for no scaffold with small pores and four scaffolds with big pores, NBA results

for twelve scaffolds with small pores and four scaffolds with big pores. BMD results were excluded from the t-test due to missing data for scaffolds with small pores.

Scaffolds with big pores (M=46.25, StD=21.34) reported statistically significant ($p<0.05$) higher BV/TV ($t=-2.66$, $p=0.029$, $d=-1.72$) than scaffolds with small pores (M=16.79, StD=13.98). The findings further revealed that scaffolds with big pores (M=22.25, StD=10.24) exhibited a significantly higher VA than scaffolds with small pores (M=10.62, StD=6.12). However, this significance was observed only when evaluating the one-sided p-value ($t=-2.28$, $p=0.026$, one-sided; $p=0.053$, two-sided, $d=-1.47$).

The results suggest rejecting the null hypothesis for BV/TV at a significance level of $\alpha=0.05$ indicating sufficient evidence supports the claim that the variables are different between the two groups and accepting H2 assuming that the pore size of a composite scaffold influences vascularisation and bone regeneration. However, the results must be carefully interpreted due to the small sample size. However, for VN and NBA, the null hypothesis was not rejected at the same significance level, indicating that there is not sufficient evidence to support the claim that the variable is different between the two groups. Further investigation is required to determine the nature of the difference between this variable and the others.

Furthermore, the results for VA suggest rejecting H0 and accepting H1 at a significance level of $\alpha=0.05$ assuming that a bigger pore size ($\geq$400 μm) of composite scaffolds improves vascularisation and bone regeneration when compared to composite scaffolds with smaller pores (<400 μm). However, the results must be carefully interpreted due to the small sample size. However, for VN and NBA, the null hypothesis was not rejected at the same significance level, indicating that there is not sufficient evidence to support the claim that the variable is different between the two groups. Further investigation is required to determine the nature of the difference between this variable and the others.

Table 5. Independent Samples Test (Pore Size).

	T-Test for Equality of Means						Cohen´s d		
					95% Confidence Interval			95% Confidence Interval	
	One-Sided p*	Two-Sided p*	Mean Difference*	Std. Error Difference*	Lower*	Upper*	Standardizer**	Lower	Upper
VN	0.245	0.491	-13.08333	17.60772	-58.34543	32.17876	23.05392	-2.077	0.994
VA	0.026	0.053	-11.63000	5.11304	-23.42069	0.16069	7.92108	-2.884	0.015
BV/TV	0.014	0.029	-29.45833	11.04948	-54.93847	-3.97820	17.11777	-3.197	-0.172
NBA	0.491	0.981	0.39917	16.87846	-35.80153	36.59987	29.23435	-1.118	1.145

*. Equal variances were assumed for all tested outcomes (p>0.05) according to Levene´s Test.

**. The denominator used in estimating the effect sizes: Cohen´s d uses the pooled standard deviation.

3.3.2 SCAFFOLD COMPOSITION

Studies using 3D composite scaffolds composed of a combination of inorganic and organic synthetic materials were included in the review (Table 1). The studies analysed a total of ten different scaffolds with various modifications (20–27). Hence, it was decided to categorise the scaffolds based on their material composition rather than the specific constituent materials employed: categorising them into simple scaffolds, composite scaffolds, and composite scaffolds with enhancements.

Independent samples tests comparing composite and non-composite materials were performed showing the following results (Table 6): VN results for five pure composite scaffolds and one non-composite scaffold, VA results for eight pure composite scaffolds and two non-composite scaffolds, BV/TV results for six pure composite scaffolds and two non-composite scaffolds, BMD results for three pure composite scaffolds and no non-composite scaffolds, NBA results for eight pure composite scaffolds and two non-composite scaffolds. Due to missing values, statistical analysis could not be performed for BMD.

There were no statistically significant differences ($p>0.05$) between pure composite scaffolds and non-composite scaffolds for VN, VA, and BV/TV. However, pure composite scaffolds (NBA: M=17.38, StD=15.08) exhibited a statistically significantly higher NBA ($t=2.70$, $p=0.031$, $d=1.02$) compared to non-composite scaffolds (NBA: M=2.99, StD=0.23) suggesting that the use of composite material may influence the newly formed bone area. For BMD, results could not be calculated due to insufficient data and had to be excluded from this analysis.

Within the data provided, the null hypothesis of no differences between group means was rejected for NBA at a significance level of $\alpha=0.05$. However, the same null hypothesis was not rejected for VN, VA, and BV/TV. Considering the small sample size and missing data these findings must be interpreted cautiously. Additional data must be collected to increase the sample size providing more insight into the relationships between variables.

Table 6. Independent Samples Test (Composite yes/no).

	T-Test for Equality of Means						Cohen´s d		
					95% Confidence Interval			95% Confidence Interval	
	One-Sided p*	Two-Sided p*	Mean Difference*	Std. Error Difference*	Lower*	Upper*	Standardizer**	Lower	Upper
VN	0.300	0.601	10.80000	19.02735	-42.02839	63.62839	17.36951	-1.601	2.773
VA	0.245	0.490	5.80250	8.02872	-12.71177	24.31677	10.15562	-1.019	2.128
BV/TV	0.166	0.333	11.95000	11.34535	-15.81108	39.71108	13.89516	-0.841	2.498
NBA	0.015	0.031	14.38625	5.33316	1.77991	26.99259	14.10409	-0.634	2.617

***. Equal variances were assumed for all tested outcomes (p>0.05) except NBA (p=0.031) according to Levene´s Test.**

Levene´s Test could not be performed for VN due to too less data.

****. The denominator used in estimating the effect sizes: Cohen´s d uses the pooled standard deviation.**

3.3.3 SCAFFOLD MODIFICATION

All included studies enhanced their scaffolds with various osteogenic and/or angiogenic factors (Table 1). Due to the diversity of enhancements employed in the included studies, the decision was made to categorise the composite scaffolds according to the number of factors added rather than the specific enhancements used: categorising them into single-factor modified composite scaffolds and dual-factor modified composite scaffolds.

The impact of scaffold modifications on vascularisation and bone regeneration was investigated with independent samples tests (t-tests). These t-tests were performed comparing modified and non-modified scaffolds (Table 7). The following results were obtained: VN results for seven modified scaffolds and six non-modified scaffolds, VA results for ten modified and ten non-modified scaffolds, BV/TV results for eight modified and eight non-modified scaffolds, BMD results for seven modified and three non-modified scaffolds, and NBA results for fourteen modified and ten non-modified scaffolds.

Scaffolds with modifications (M=48.57, StD=25.61) reported a statistically significant ($p<0.05$) higher number of VN than the pure scaffolds (M=22.00, StD=16.15). However, this significance was observed only when evaluating the one-sided p-value ($t=2.19$, $p=0.026$ (one-sided), $p=0.051$ (two-sided), $d=1.22$). The findings further show that scaffolds with modification (M=31.74, StD=25.60) reported a statistically significant ($p<0.05$) bigger VA ($t=2.58$, $p=0.025$, $d=1.15$) than pure scaffolds (M=9.38, StD=9.88). Additionally, independent samples t-tests were performed to evaluate new bone formation by testing study results of BV/TV, BMD, and NBA. The test showed that scaffolds with modification (BV/TV: M=35.58, StD=23.67; BMD: M=0.55, StD=0.31; BMD: M=0.55, StD=0.31; NBA: M=47.24, StD=30.23) reported statistically significant ($p<0.05$) higher values for BV/TV and NBA ($t=2.28$, $p=0.039$, $d=1.14$, $t=3.52$, $p=0.002$, $d=1.31$ respectively) than pure scaffolds (BV/TV: M=13.42, StD=14.00; BMD: M=0.44, StD=0.53; NBA: M=14.50, StD=14.62).

The results suggest rejecting H0 for VA and BV/TV at a significance level of $\alpha=0.05$, and for NBA at a significance level of $\alpha=0.01$, indicating that there is sufficient evidence to support the claim that the variables are different between the two groups and accepting H2 assuming that the modification of composite scaffolds influences vascularisation and bone regeneration. Furthermore, the results for VN suggest rejecting H0 and accepting H1 at a significance level of $\alpha=0.05$ assuming that the modification of composite scaffolds improves vascularisation and bone regeneration. However, the results must be carefully interpreted due to the small sample size.

For the BMD, the null hypothesis was not rejected at the same significance level, indicating that there is not sufficient evidence to support the claim that the variable is different between the two

groups. Further investigation is required to determine the nature of the difference between this variable and the others.

Table 7. Independent Samples Test (Modification yes/no).

	T-Test for Equality of Means						Cohen´s d		
					95% Confidence Interval			95% Confidence Interval	
	One-Sided p*	Two-Sided p*	Mean Difference*	Std. Error Difference*	Lower*	Upper*	Standardizer**	Lower	Upper
VN	0.026	0.051	26.57143	12.14240	-0.15381	53.29667	21.82516	-0.006	2.2396
VA	0.012	0.025	22.36000	8.67643	3.38782	41.33218	19.40108	0.186	2.092
BV/TV	0.019	0.039	22.15250	9.72316	1.29840	43.00660	19.44632	0.057	2.188
BMD	0.341	0.682	0.11143	0.26194	-0.49260	0.71546	0.37959	-1.075	1.645
NBA	0.001	0.002	32.74171	9.30813	13.31478	52.16865	25.04854	0.397	2.193

*. Equal variances were assumed for all tested outcomes (p>0.05) except for VA (p=0.038) and NBA (p=0.029) according to Levene´s Test.

**. The denominator used in estimating the effect sizes: Cohen´s d uses the pooled standard deviation.

Independent samples t-tests compared single-modified and dual-modified scaffolds (Table 8). In the population studied, the following results were obtained: VN results for five single-modified and two dual-modified scaffolds, VA results for eight single-modified and two dual-modified scaffolds, BV/TV results for six single-modified and two dual-modified scaffolds, BMD results for five single-modified and two dual-modified scaffolds, NBA results for ten single-modified and four dual-modified scaffolds.

Scaffolds with two enhancements (VN: M=85.00, StD=7.07; BV/TV: M=63.00, StD=14.14; NBA: M=81.63, StD= 15.88) reported statistically significant (p>0.05) higher VN (t=-9.19, p=<0.001, d=-7.69), higher BV/TV (t=-2.51, p=0.046, d=-2.05), and higher NBA (t=-3.89, p=0.002, d=-2.30) than scaffolds with one enhancement (VN: M=34.00, StD=6.52; BV/TV: M=26.43, StD=18.53; NBA: M=33.48, StD=22.37). The findings further revealed that scaffolds with two modifications (M=60.55, StD=36.13) exhibited a significantly higher VA than scaffolds with one modification (M=24.54, StD=18.96). However, this significance was observed only when evaluating the one-sided p-value (t=-2.08, p=0.035, one-sided; p=0.071, two-sided, d=-1.65).

The results suggest rejecting the null hypothesis for BV/TV at a significance level of α=0.05 and for other variables (VN, NBA) at a significance level of α=0.01 indicating that there is sufficient evidence to support the claim that the variables are different between the two groups and accepting H2 assuming that the number of osteogenic factors in the modification of composite scaffolds influences vascularisation and bone regeneration. Furthermore, the results for VA suggest rejecting H0 and accepting H1 at a significance level of α=0.05 assuming that dual-factor modified composite scaffolds improve vascularisation and bone regeneration compared to single-factor modified ones. However, the results must be carefully interpreted due to the small sample size. However, for the BMD, the null hypothesis was not rejected at the same significance level, indicating that there is not

sufficient evidence to support the claim that the variable is different between the two groups. Further investigation is required to determine the nature of the difference between this variable and the others.

Table 8. Independent Samples Test (Number of Modifications).

	T-Test for Equality of Means						Cohen´s d		
					95% Confidence Interval			95% Confidence Interval	
	One-Sided p*	Two-Sided p*	Mean Difference*	Std. Error Difference*	Lower*	Upper*	Standardizer**	Lower	Upper
VN	<0.001	<0.001	-51.00000	5.54977	-65.26615	-36.73385	6.63325	-12.563	-2.796
VA	0.035	0.071	-36.00375	17.28041	-75.85244	3.84494	21.85818	-3.348	0.133
BV/TV	0.023	0.046	-36.56667	14.59030	-72.26784	-0.86549	17.86939	-3.952	-0.033
BMD	0.314	0.627	-0.14500	0.28051	-0.86608	0.57608	0.33528	-2.072	1.248
NBA	0.001	0.002	-48.13800	12.38631	-75.12544	-21.15056	20.93668	-3.744	-0.796

*. Equal variances were assumed for all tested outcomes (p>0.05) according to Levene´s Test.

**. The denominator used in estimating the effect sizes: Cohen´s d uses the pooled standard deviation.

3.4 RESULTS: RISK OF BIAS

The analysis results show three studies with at least one high-risk item (Figure 2). Min Z. et al. (24) failed to quantify their vascularisation and bone formation results (VN, VA, BMD, BV/TV, NBA) and reported “Microfil” results for a control group without indicating the number of animals in that group. In the study of Kuttappan S. et al. (27) VN, BV/TV, and BMD were not reported, and VA was given solely in fold increase without a relation. Furthermore, the number of rats in total and per group was not stated. In the study by Wang Y. et al. (25), all rats received both scaffolds, polylactic acid-hydroxyapatite (PLA-HA) with vascular endothelial GF (VEGF) placed into the left calvarial defect, and PLA-HA without VEGF in the right calvarial defect causing a non-random allocation. However, it can be assumed that the housing was random as each rat was incorporated into both groups. Moreover, the study failed to provide NBA results, and differences between reported numbers and numbers in graphics for VN and VA were detected. Other three studies (20–22) failed to report results for BMD (20), VN and BMD (21), VN, BV/TV, and BMD (22). However, each of these studies reported at least one vascularisation outcome and one bone formation outcome and therefore was not considered high-risk for selective outcome reporting.

Six of the analysed studies (20–25) created two defects in each rat, four of them (20,21,23,24) implanted the same scaffolds in both defects and one study (25) both of their scaffolds in each rat. It is unclear whether these findings influenced the risk of bias in these studies. The study by Wang G. et al. (22) was the only one to report implanting their scaffolds randomly for each defect and therefore was considered low risk for this category.

Study	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	Overall
Qi X. et al. 2017	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Unclear	Not applicable
Min Z. et al. 2015	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	High	High	Not applicable
Kuttappan S. et al. 2018	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear	Low	High	Low	Not applicable
Sun T.W. et al. 2017	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Unclear	Not applicable
Wang Y. et al. 2023	High	Low	High	Low	Unclear	Unclear	Unclear	Low	High	High	Not applicable
Li S. et al. 2019	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Unclear	Not applicable
Tu C. et al. 2020	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low	Not applicable
Wang G. et al. 2017	Low	Low	Unclear	Unclear	Unclear	Unclear	Unclear	Low	Low	Low	Not applicable

D1: Random sequence generation
D2: Baseline characteristics
D3: Allocation concealment
D4: Random housing
D5: Blinding of caregivers and investigators
D6: Random outcome assesment
D7: Blinding of outcome assesors
D8: Incomplete outcome data
D9: Selective outcome reporting
D10: Other sources of bias

Fig. 2. Risk of Bias in the Animal Studies.

4 DISCUSSION

This review aimed to find common factors influencing a scaffold´s vascularisation and bone regeneration ability in the context of oral and maxillofacial surgery. As far as is known, no systematic review has been conducted before to investigate the vascularisation and bone regeneration of composite scaffolds in rodents using the innovative “Microfil” perfusion and micro-CT techniques. To find the ideal scaffold for successful vascularisation and bone regeneration in critical-size defects, the scaffold should have the following features: (1) Biocompatibility; (2) Easy application in defect area; (3) Osteoconductivity, and osteoinductivity; (4) Mechanical properties similar to natural bone; (5) Suitable surface morphology for cell attachment and appropriate pore structure allowing a dynamic extracellular matrix promoting cell proliferation and differentiation (6) Suitable structure allowing vessel ingrowth and supporting vascularisation; and (7) Controlled degradation rate according to formation rate of natural bone without toxic byproducts (28,29). Composite scaffolds combining organic and inorganic components resemble natural bone more closely (13); thus, other scaffolds were excluded as part of the investigations.

Bone healing is a multifaceted process including cell migration, proliferation, formation of extracellular matrix, and angiogenesis (30). In the context of skeletal development and repair, angiogenesis plays a crucial role: by restoring blood flow, it enables the delivery of nutrients and mobilises autologous cells to repair defects. The stages of angiogenesis include basement membrane degradation and the migration of endothelial cells which are regulated by angiogenic factors such as

fibroblast GFs (FGFs), angiogenin, transforming GF, tumour necrosis factor, and VEGF. Vascular and angiogenesis deficiencies will result in delayed or non-union. Consequently, incorporating angiogenic factors into bone tissue engineering is critical for improving bone repair strategies. Therefore, combined delivery of osteogenic and angiogenic factors is essential for bone growth and regeneration (31).

Even though various studies have investigated possible scaffold materials and designs, the incorporation of angiogenic and osteogenic factors, none of them has succeeded in building an ideal scaffold (28). Until today, addressing larger bone defects remains challenging due to the limited vascular network within tissue-engineered bone substitutes (28,31).

Several scaffold materials and designs in combination with various exogenous factors and stem cells make it complicated to identify efficient scaffolds for successful vascularisation and bone regeneration. By employing the dual imaging technique combining "Microfil" perfusion and micro-CT imaging this review increases comparability among studies allowing us to identify factors influencing vascularisation and bone regeneration.

4.1 DISCUSSION: STUDY SELECTION

According to the electronic search, only eight studies met the inclusion criteria for this review. Among 548 studies only forty-eight used "Microfil" perfusion to assess a scaffold´s vascularisation properties. This suggests that the innovative dual imaging technique, combining "Microfil" perfusion and micro-CT, has not been widely utilised on a larger scale. The need for additional equipment and its sensitivity to technique may limit the number of studies conducted using this method. Forty-eight studies were further assessed for eligibility, of which twenty-six did not involve surgical procedures in the rodents' skull or mandible and, therefore, were excluded due to their lack of relevance to oral and maxillofacial surgery research. Moreover, sixteen studies assessed for eligibility used scaffold materials other than composite decreasing the number of included studies even further (Fig. 1).

Those findings let us conclude, that additional *in vivo* experiments using "Microfil" perfusion to evaluate the vascularisation properties of composite scaffolds implanted into the calvarial bone are necessary to identify the ideal scaffold material, design, and modifications for optimal vascularisation and bone regeneration in the field of oral and maxillofacial surgery.

4.2 DISCUSSION: STUDY CHARACTERISTICS

4.2.1 ANIMALS

The heterogeneity of utilised rats in evaluated studies, including age, gender, and strain, may impact the vascularisation and bone regeneration results. A systematic review of critical-size defect models found higher new bone formation in bigger defects (9 mm in diameter), likely since younger

adult rats (3 months old) were used in those experiments (compared to other smaller defects in more mature rats (4-6 months old)) (32). It was recommended to avoid younger rats due to the potential for spontaneous bone repair in large defects and instead opt for rats aged at least 16 weeks (32,33). Most studies included in this analysis used rats with an age of 8-13 weeks (20–22,24–26) potentially impacting the bone repair outcomes.

Moreover, the gender and strain of the utilised rats could be of importance in the amount of new bone created in a critical-size rat calvarial defect (32,34). Female gender was found to be a risk factor for impaired bone healing in middle-aged rats (34). However, none of the included studies performed their experiments on female rats. Once clinical studies confirm such findings for humans, female rats should be included in future experimental studies.

Even though rat strains differ to some extent in behaviour, hormone levels, and antioxidant status (35), their impact on new bone formation and vascularisation remains unclear. Until further research confirms the influence of rat strains on new bone formation and neovascularisation outcomes, both SD and Wistar rats, can be considered suitable candidates for experimental studies in bone regeneration.

4.2.2 SAMPLE SIZE, STUDY GROUPS, AND DEFECT FEATURES

Differences in defect sizes have shown to be critical, with defects of 5 and 8 mm in diameter most encountered. Defects measuring 5 mm offer to create two defects in the same adult rat calvaria and allow more efficient use of animals by increasing the number of defects, whereas 8 mm defects must be performed in a central location involving the midsagittal suture. A systematic review could show that 5 mm defects very rarely (1.6%) healed completely with newly formed bone and therefore were considered critical-size defects. Unexpectedly, the extent of new bone formation varied greatly among studies with identical defect sizes, showing no correlation with size. Regarding the anatomic location of the defect, there was no difference in new bone formation comparing central and bilateral 5 mm defects at 1- and 3-month follow-ups. An advantage of 5 mm defects is that the control and experimental sites are in the same animal lowering the risk of bias caused by variability among the studied animals. Additionally, an injury to the sagittal sinus causing complications is less likely, and overall fewer animals are needed for an experiment. However, certain angiogenic and/or osteogenic factors added in the form of gels or liquids locally to the experimental site may spread to the nearby control site (32). Considering both advantages and disadvantages, it is recommended to employ bilateral 5 mm defects.

4.2.3 TIMING OF “MICROFIL” PERFUSION, MICRO-CT, AND HISTOLOGY

The different timing of “Microfil” perfusion and micro-CT after implantation (varying from to) of the included studies may have influenced this study's results since studies (26,27) showed

greater newly formed bone area as the implantation period increased. Moreover, the only included study performing "Microfil" perfusion at two different time points (4 and 8 weeks) presented increased vascularisation results (VN, VA) with increased evaluation time (25). However, a systematic review demonstrated that there was not always a considerable difference in new bone formation comparing 1 and 2-month evaluation periods. In the case of high new bone formation on final evaluation, a trend was already seen in early evaluation points (32).

4.2.3 VASCULARISATION ANALYSIS

Included studies reported considerably improved neovascularisation when osteogenic factors were added (21–25,27), and significantly greater neovascularisation when two osteogenic factors were added (23,26,27) with microvessels growing along the round edge of scaffolds (23). There was no difference observed between dual-factor loaded groups (bone morphogenetic protein 2 (BMP-2) with VEGF or FGF2) (27), but with them also the single-modified VEGF group had significantly higher blood vessel formation compared to other groups (27). However, significantly higher VA was only demonstrated in dual-factor (BMP-2 with VEGF or FGF2) loaded groups with no difference between them (27). The study by Wang Y. et al. (25) further demonstrated that VA, in the area of implanted modified scaffold (PLA-HA added with VEGF), gradually increased with increasing time compared to the blank scaffold (PLA-HA). Moreover, two studies (20,21) demonstrated that the new VA was significantly higher for the blank composite scaffolds (mesoporous bioactive glass (BG)-poly(lactide-co-glycolic acid) (PLGA); chitosan (CS)-HA nanowires (NWs) and CS-HANW added with magnesium silicate (MS) nanosheets) compared to non-composite scaffolds (PLGA; CS). CS-HANW added with MS nanosheets further showed significantly greater VN and VA than CS-HANWs and CS groups (20). The positive effect of MS on vascularisation was confirmed by Wu T. et al. (36) who reported increased osteogenic differentiation and enhanced angiogenesis using a novel composite porous scaffold combining calcium MS with silk fibroin and graphene oxide. Similarly, Yang J. et al. (37) found micro-RNA-146a-loaded MS nanospheres having dual osteogenic and immunoregulatory effects, promising for treating oral-maxillofacial bone defects.

4.2.5 NEW BONE FORMATION ANALYSIS

Barely new bone formation was seen in blank composite scaffolds, specifically, mesoporous BG combined with poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (23) and tricalcium phosphate (TCP)-HA (22), and control groups (26). However, according to Li S. et al. (21), the blank composite scaffold (mesoporous BG-PLGA) resulted in significantly greater new bone formation and mineralisation compared to the simple non-composite scaffold (PLGA) and control groups, which showed no significant difference. Nevertheless, the highest new bone formation and mineralisation

in the mentioned study (21) were found in the composite scaffold group modified with bioactive lipid FTY720 (FTY) (mesoporous BG-PLGA added with FTY).

4.3 DISCUSSION: STATISTICS

Not all studies reported the same parameters for vascularisation, bone formation analysis, and scaffold structure. In addition to missing data, challenges arose in grouping due to the variety of scaffold types and study design. Consequently, depending on the parameter tested, some studies were excluded from the statistical analysis resulting in a smaller number of included values. This could have increased the potential for analysis errors, thus diminishing the significance of the presented findings. The current investigations underline the importance of a uniform study design, employing the same parameters in vascularisation and bone formation analysis, and scaffold structure reporting. Future studies should include all relevant scaffold characteristics and vascularisation and bone regeneration results to improve comparability and inclusiveness among available studies.

To evaluate the results in the context of statistical hypothesis testing, two-sided p-values are preferred as they provide a more comprehensive evaluation and broader interpretation of deviations in either direction from the null hypothesis. While in this analysis most variables exhibited statistical significance based on two-sided p-values, for VN ($p=0.026$ one-sided, and $p=0.051$ two-sided), when comparing scaffolds with modifications and simple scaffolds, the two-sided p-value was not statistically significant at $p<0.05$ (Table 7). A similar finding was made for VA ($p=0.035$ one-sided, and $p=0.071$ two-sided) when investigating differences between dual-factor and single-factor modifications (Table 8), and for VA ($p=0.026$ one-sided, and $p=0.053$ two-sided) when analysing if pore size influences the vascularisation outcomes (Table 5). This can be explained by the small number of included studies, along with selective outcome reporting of some of those studies leaving us with a small number of assessable outcomes.

Even though the results of the Shapiro-Wilk test indicate that the data was mostly normally distributed for all independent variables ($p>0.05$) except for “porosity” ($p<0.05$), due to the small sample size, it cannot be concluded that the sample has a normal distribution. Indeed, the sample does not have a normal distribution. The inquiry as to the distribution of the underlying population remains unanswered. Additionally, the reliability of Pearson correlation coefficients for all variables may be compromised due to the small sample size. Therefore, further investigation is necessary to determine the true nature of the relationships between variables.

4.3.1 SCAFFOLD DESIGN

While the pore size indicates the diameter of empty spaces within a scaffold, porosity shows the proportion of these voids (38). Even though the porosity of cortical bone is approximately 5-13% (compared to 30-90% in cancellous bone), a scaffold interconnected porosity of 90% with pores of at

least 100 μm has been recommended. This should ensure cellular interaction, transport of nutrients and wastes, the ingrowth of bone and its vascularisation, and finally better osteointegration (39,40). However, greater porosity decreases scaffold mechanical strength, necessitating a balance between these opposing factors (39).

Pore size has the potential to influence the rate and extent of vascularisation and bone regeneration (41–43). The reported ideal pore size required for optimal vascularisation and bone regeneration varies from 100 to 800 μm, leading to controversy on the topic (19,44,45). Larger pore sizes and higher porosity can lead to a faster rate of neovascularisation, which can enhance bone regeneration (42). Pores with a diameter of 150-800 μm promote bone and blood vessel growth, whereas pores sized 10-100 μm support blood capillary development, nutrient exchange, and waste excretion (45) underlining the great influence of scaffold microstructure on cell functions in tissue engineering (46).

Regarding the scaffold dimension, although one study (25) implanted a flat membrane instead of a scaffold, it was included in parts of this analysis. As the study met all other inclusion criteria, including the 3D analysis with "Microfil" perfusion and micro-CT, it was valuable for this analysis.

Results revealed a tendency toward increased vascularisation and bone regeneration with larger pore sizes (≥400 μm). However, only two variables showed statistically significant results (two-sided p=0.029 for BV/TV, one-sided p=0.026 for VA), highlighting the need for additional investigation (Table 5).

It should be emphasised that two studies (23,25) were excluded from this statistical comparison for not reporting a pore size (25) and for employing a hierarchical scaffold with macro- (450–900 μm) and micropores (20–50 μm) (23). Thus far, hierarchical scaffolds combining micro- and macropores have shown great promise in improving cell activity, attachment, and bone ingrowth, proposing their potential superiority in bone tissue engineering. To achieve cell responses similar to natural bone, a high porosity ranging from 60-90% combined with macro- (200–800 μm) and micropores (<10 μm) was proposed (38). Micro- and macropore structures not only enhance cell activity within the scaffold but also influence cell attachment, proliferation, and bone ingrowth due to their rough surface. Macropores could stimulate cell penetration whereas micropores improve ion exchange and bone protein adsorption (38). An experimental study investigating hierarchical β-TCP scaffolds with fibrous micropores demonstrated higher vessel formation and significantly increased BV/TV (47). Similarly, nanofibrous poly-L-lactic acid (PLLA) scaffolds with both micro- and macropores demonstrated enhanced cell proliferation and infiltration, highlighting the significant impact of fibre and pore size on biological scaffold properties (46).

A recent systematic review (19) has emphasised the potential of novel bi-layered scaffolds with gradient pore size and properties similar to natural bone, showing improved biological outcomes

compared to single-layer structures. The first layer is dense and prevents connective tissue penetration while enhancing the proliferation of the fibrous layer and aiding in wound healing. The second layer is loose and has sufficient pore size and porosity to facilitate cell migration, osteoblast differentiation, and vascularisation.

Adapting scaffold morphology, such as pore size and porosity, potentially enhances new vessel formation and bone regeneration possibly eliminating additional need for angiogenic and osteogenic factors. Overall, the presented findings highlight the importance of properly designed scaffold structures with optimal porosity, and micro- and macrostructure in advancing bone tissue engineering techniques. Considering the potential of hierarchical pore structure, additional studies exploring such scaffold designs are needed.

4.3.2 SCAFFOLD COMPOSITION

The analysis revealed the diversity of composite materials used in the included studies. Therefore, a direct comparison between all scaffold materials was impossible and potentially impacted the presented study results. Different composite scaffold materials have particular advantages and disadvantages affecting vascularisation and bone regeneration outcomes (48,49). While metals like titanium, nickel-titanium, or strontium (Sr) possess great biocompatibility and superior strength, BG and ceramics such as HA have superior osteoconductive and osteoinductive properties. Ceramics possess a similar composition to host bone mineral content, however, when used alone they are brittle and may show inappropriate degradation. Polymers are biodegradable and often incorporate biofunctional molecules on their surface. While natural polymers like CS or gelatine can be derived from ECM showing superior biocompatibility and low toxicity, synthetic polymers like PLA and PLGA provide enhanced control over physical properties. However, synthetic polymers are often hydrophobic lacking cell recognition sites. Both natural and synthetic polymers, generally miss mechanical properties for load-bearing (1). The range of available scaffold materials, combining different materials, is enormous as seen in this analysis. Additional studies must be included to effectively compare individual scaffold materials in the context of vascularisation and bone regeneration outcomes.

The presented findings demonstrate a difference between vascularisation and bone regeneration outcomes of composite and non-composite scaffolds; although, significantly different only for the NBA (Table 6). Sun T.W. et al. (20) and Li S. et al. (21) both showed superior outcomes for composite scaffolds in all tested variables compared to non-composite scaffolds. Current research suggests that composite scaffolds offer advantages by correcting the limitations of other biomaterials like polymer, ceramic, or metal. By combining two or more biomaterials, various studies found that composite scaffolds can fit the requirements of the targeted tissue much better than a single material

(43,50). These scaffolds are designed to not only fit the mechanical needs of the implantation site but also match biological requirements (43).

A wide range of synthetic polymers serve as biomaterials, with PLA, polycaprolactone, and PLGA being the most frequently employed due to their great biocompatibility and biodegradability. Also, natural polymers and bioceramic materials like CS, collagen, and HA have been used and combined with synthetic, natural polymers, and bioceramic materials (43). Expanding upon the recommendation by Reddy M.S.B. et al. (50), this study emphasises the importance of blending natural and synthetic biopolymers to create scaffolds tailored for tissue replacement addressing all clinical requirements. As part of this investigation, studies that focused on composite scaffolds lacking a combination of natural and synthetic materials were excluded.

As for this investigation, more studies comparing composite and non-composite scaffolds must be included to conclude whether composite scaffolds exhibit superior vascularisation and bone regeneration properties compared to non-composite scaffolds in critical-size defect repair.

4.3.3 SCAFFOLD MODIFICATION

To create the perfect scaffold, scientists have researched different ways of enhancing angiogenic and osteogenic scaffold properties: GF loading, stem cell loading, small-molecule drug loading, and their combinations. Such modifications have been demonstrated to be important in enhancing cell adhesion, proliferation, and differentiation (30,51). Exogenous GFs can activate cellular responses, promoting angiogenesis, cell migration, proliferation, and stem cell recruitment (30). In this context, modifications refer to angiogenic and osteogenic factors, including GFs (BMP-2, FGF2, VEGF), stem cells (bone marrow-derived mesenchymal stem cells (BMSCs), adipose-derived stem cells (ASCs)), small-molecule drugs (dimethyloxalylglycine (DMOG), FTY), and scaffold treatments (electromagnetic fields (EMF)), which were added to a scaffold to enhance its vascularisation and bone regeneration. This review differentiated between scaffolds with modifications, distinguishing between scaffolds with single- and dual-factor loading, and without modifications.

According to the statistical evaluation, single- and dual-modified composite scaffolds improved neovascularisation and bone regeneration in critical-size rat calvarial defects compared to non-modified scaffolds yielding statistical significance for all variables except for BMD (Tables 7). This may be explained due to the small number of reported BMD results: only three studies (23,25,26) including those results in their analysis.

The presented results are confirmed by the individual studies included in this systematic review (21–27) and further supported by a systematic review conducted by Fiorillo L. et al. in 2021

(52): the clinical situation significantly improved when GFs were added, showing increased bone thickness and height, and improved quality of life and postoperative time.

Studies further verified the impact of stem cell loading (22,28,53–55): BMSCs expressing BMP-2, endothelial progenitor cells (EPCs) expressing VEGF and PDGF, along with ASCs play crucial roles in upregulating angiogenic and osteogenic gene expression, and thus new vessel and bone formation (56,57). The proliferation and osteogenic differentiation of BMSCs were additionally enhanced by EMF treatment, allowing for greater neovascularisation and bone formation (26), confirmed by an investigation by Li W. et al. (58). Similarly, the combination of EMF and VEGF has demonstrated the potential to be efficient in increasing angiogenesis and osteogenesis in the future (59).

Moreover, multiple small-molecule drugs like DMOG (23,24) and FTY (21) show promise for bone regeneration and neovascularisation due to their stability, short action period, and cost-effective production (60,61). While FTY was able to boost type H vessel formation successfully linking angiogenesis and osteogenesis (21), DMOG could stabilise the hypoxia-inducible factor 1 alpha pathway and activate VEGF inducing a proangiogenic effect, enhancing bone regeneration and vessel growth (23,62).

The presented statistical results demonstrate that the dual-factor loading of a composite scaffold even further enhances vascularisation and bone regeneration compared to a single-factor modification (Table 8). These results confirm what was found in some of the individual studies included in this systematic review (23,26,27). Especially, the combined delivery of either VEGF (27,31,63), FGF-2 (64,65) or insulin-like GF-1 (66,67) together with BMP-2 has shown promise in enhancing scaffold effectiveness in bone tissue engineering. Also, a combined loading with platelet-rich GFs and BMSCs demonstrated superior bone regeneration results compared to single-loaded and unloaded scaffolds in a rabbit calvarial model (68). Additional clinical animal studies could demonstrate superior bone repair outcomes in dual stem cell loaded groups: He Y. et al. (56) researched the combined delivery of ASCs and EPCs, and Yu H. et al. (57) investigated the dual delivery of EPCs and BMSCs. Furthermore, co-delivery platforms incorporating multiple angiogenic and osteogenic factors with optimised concentrations, regulated release, and independent action have been suggested to avoid various side effects associated with high-concentration loading of a single factor such as BMP-2 (30,65,69–72).

Since the mentioned enhancements affect vascularisation and bone regeneration success differently, the advantages and disadvantages of specific enhancements and their combinations must be further investigated. As far as is known, no such systematic review has been published and should be conducted in the future to confirm the superior angiogenic and osteogenic effects of dual-factor scaffold loading.

4.4 DISCUSSION: RISK OF BIAS

The risk of bias in the individual studies was low or unclear for all items of the evaluated studies except for three studies (24,25,27) which displayed high risk in at least one category. One of them (25) even exhibited a reporting error between reported VA and VN results in text versus in their graphic. Other items with a high risk of bias did not report all relevant vascularisation and bone regeneration results in detail (24,25,27) reducing sample sizes of this statistical analysis which adversely impacted its power. Nevertheless, most studies (20–23,26) did not raise concerns for an elevated risk of bias.

Furthermore, regarding all included studies around half of the assessed items were not possible to evaluate due to missing information in the reports, as none of the analysed studies specified the procedure performed for the allocation concealment, random housing, and blinding of caregivers and investigators. However, without any contradictory indications, laboratories likely employ systems ensuring blinding and randomisation.

It is necessary for future studies to implement a clear way of reporting including all factors influencing the risk of bias. Only in that case any doubts about risks of bias can be erased and properly assessed.

5 RECOMMENDATIONS FOR FUTURE RESEARCH

For uniform reporting in future animal studies evaluating the vascularisation and bone regeneration of 3D bone scaffolds employing “Microfil” perfusion, researchers are encouraged to adhere to the following guidelines. Following these guidelines not only aids in lowering the potential risk of bias but also makes results comparable among studies. The subsequent recommendations are based on the results obtained in this systematic review in accordance with the SYRCLE’s risk of bias tool (17):

1. Researchers are encouraged to perform their experiments using **mature male and female SD or Wistar rats**, **housed randomly**.
2. It is recommended to **divide the study samples randomly** into the following groups: control group **(no scaffold)**, simple scaffold group **(non-composite)**, composite scaffold group **(composite)**, composite scaffold groups added with one angiogenic or osteogenic factor **(enhanced composite)**, and further enhanced composite groups added with two or multiple angiogenic and osteogenic factors **(double/multiple enhancements)**. Researchers are further encouraged to experiment with different scaffold designs, comparing scaffolds with big pores **(pores ≥ 400 µm)**, and scaffolds with a combined pore structure of micro- and macropores **(mixed pores)**.

3. To increase the number of investigated defects while minimising animal usage, it is advised to create **two defects,** each **with a diameter of 5 mm,** per rat. An exact **sample size** and **defect number** should be indicated for each group specifying the number of defects investigated with "Microfil" perfusion and micro-CT. It is further important to assign the groups **randomly for each defect** and indicate that in the description of the study design.
4. "Microfil" perfusion and micro-CT should preferably be performed at multiple time points: **4-, 8-, and 12-weeks** post-implantation. If researchers decide to conduct a single investigation, "Microfil" perfusion and micro-CT should be performed **at 8 weeks** post-surgery. To gain additional information researchers are encouraged to proceed with a histological analysis as "Microfil" perfusion and micro-CT leave samples intact for further study.
5. To evaluate and compare study results it is recommend performing a quantitative analysis including the following parameters: **VN** (exact number), **VA** (in %), **BV/TV** (in %), **BMD** (in g/cm^3), and **NBA** (in %). Furthermore, **porosity** (in %), **pore sizes** (in μm), and **pore structure** should be reported for all scaffolds transplanted. The results should be reported in numbers avoiding vague graphics.
6. To avoid risk of bias researchers should make sure to report the following data and proceed accordingly: perform a **random allocation sequence** (random number generator), use the **same baseline criteria** for all rats (sex, age, weight), perform an adequately **concealed allocation** to the different groups (third-party coding), provide **random housing** (cages placed randomly in the room), **blind** caregivers and investigators (coded cage labels etc.), assess the **outcome randomly** (random number generator), **blind** the outcome assessor (same outcome assessment methods for all groups with random selection), report any **incomplete outcome data** (missing rats in analysis, reasons for missing outcome data), report **all expected outcomes**, and avoid any **other sources of bias** (analysis and reporting errors, rats added to replace drop-outs, inappropriate funding etc.) (17).

6 CONCLUSION

Despite significant progress in the field of bone tissue engineering, clinicians still face challenges in achieving vascularisation and bone regeneration for critical-sized calvarial defects.

This systematic review analysed the effect of 3D composite scaffolds on neovascularisation and new bone formation in critical-sized calvarial defects in rodents using "Microfil" perfusion and micro-CT.

According to the limitations of this research (small number of analysed studies, their heterogeneity, and a high risk of bias in three of those studies), it was found that composite

scaffolds, along with modifications (such as angiogenic and/or osteogenic factors) and larger pore sizes (≥400 μm), may positively influence superior vascularisation and bone regeneration outcomes. Further research is needed to adapt scaffold morphology on micro- and macropores, find the best composition of scaffold material and in advance to reduce the need for adding expensive angiogenic and osteogenic factors. Due to the high risk of bias, recommendations for future *in vivo* studies, comparing scaffold materials, designs, and modifications, were addressed for the researchers.

7 CONSENT FOR PUBLICATION

Not applicable.

8 STANDARDS OF REPORTING

PRISMA guidelines and methodology were followed in this study.

9 FUNDING

None.

10 CONFLICTS OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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