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Systematic literature review of *in vivo* rat femoral defect models using biomaterials to improve the induced membrane technique: a comprehensive analysis

Marc Saab[1,2], Cédric Zobrist[3], Nicolas Blanchemain[2], Bernard Martel[3] and Feng Chai[2]

[1]CHU Lille, Orthopaedic and Traumatology Department, Hôpital Roger Salengro, Lille, France
[2]University of Lille, INSERM, CHU Lille, U1008 – Advanced Drug Delivery Systems and Biomaterials, Lille, France
[3]University of Lille, CNRS, INRAE, Centrale Lille, UMR 8207 – UMET – Unité Matériaux et Transformations, Lille, France

Correspondence should be addressed to M Saab Email marc.saab@outlook.com

- *Purpose:* The aim of this study was to conduct a systematic literature review analyzing the results of *in vivo* rat femoral defect models using biomaterials for improving the induced membrane technique (IMT).
- *Methods:* Following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines, the PubMed, Embase, and Web of Science databases were searched. Inclusion criteria were studies reporting results of the IMT in *in vivo* rat femoral critical-sized defect models using a biomaterial possibly combined with molecules. Methodologic quality was assessed with the Animal Research: Reporting *In Vivo* Experiments guidelines.
- *Results:* Twenty studies met the inclusion criteria. Femoral stabilization with plate and screws was the most frequent. Histologic, biomechanical, and/or radiologic analyses were performed. In two-stage strategies, the PMMA spacer could be associated with bioactive molecules to enhance IM growth factor expression and improve bone formation. Modulating the roughness of spacers could increase IM thickness and accelerate its formation. In one-stage strategies, human tissue-derived membranes combined with bone grafting achieved bone formation comparable to a standard IMT. All calcium phosphate grafts seemed to require a functionalization with growth factors or bone marrow mononuclear cells to improve outcomes compared with non-functionalized grafts.
- *Conclusion:* This systematic review described the main parameters of the *in vivo* rat femoral defect models using biomaterials to improve the induced membrane technique. Although the studies included had several methodological limitations that may limit the scope of these conclusions, one- and two-stage strategies reported promising results with biomaterials to improve the IMT.

Keywords: induced membrane technique; PMMA; calcium phosphate spacer; artificial membrane; polymers; human tissue-derived membranes; femoral defect; bone defect

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Introduction

The management of critical bone loss in orthopedic and trauma surgery can be managed by the induced membrane technique (IMT), developed by A-C Masquelet (1). It consists of placing a PMMA spacer into the bone defect. A foreign-body reaction leads to the formation of an induced membrane (IM) around the spacer that will serve as a receptacle for a bone autograft. Despite the high success rate (up to 90%) of this technique, the IM is formed over a fairly long period of time before bone grafting (usually from 6 weeks to 3 months), it requires two surgical interventions during two prolonged hospitalizations (2). The relative immobility of the patient for several weeks has not only physical consequences, like amyotrophy and joint stiffening, but has also socio-economic consequences with a delay in returning to personal and professional activities. The formation of the IM depends on the patient and the pathological condition that could decrease its quality (age, smoking, comorbidities, associated infectious diseases, localization of the bone defect, loss of soft tissue coverage). The failure of the IMT may be due to an insufficient remodeling of the extracellular matrix around the spacer, sometimes leading to friable and fragile IM intraoperatively (2).

Animal models are widely used to understand and enhance the highly complex biological and cellular mechanisms that lead to IM formation. Many studies evaluated biomaterials alone or functionalized with molecules or cells, in order to reduce the IMT to a single-stage procedure or to improve the two-stage procedure with another spacer or graft material. Through *in vivo* assessment, new biomaterials that enhance the IMT could be selected and then allow, from a clinical perspective, a faster and higher recovery rate. The *in vivo* rat femoral defect is the most frequent model studied in this field (3). Indeed, the breeding, husbandry, and surgical procedures on rats are faster and easier with significantly reduced costs compared to larger models. The femur has a cylindrical shape allowing a more secure osteosynthesis and better soft tissue coverage compared to the tibia (4, 5). Thus, the objective of this systematic literature review was to provide a comprehensive analysis of the *in vivo* rat femoral defect models used to evaluate the biomaterials and/or molecules used to improve the IMT. The main hypothesis was that this systematic review could identify which biomaterials and/or molecules provided the most favorable results.

Materials and methods

Search strategy

A search was performed on the PubMed (Medline), Embase, and Web of Science databases in December 2022 while following the PRISMA (Preferred Reporting Items for Systemic Reviews and Meta-Analysis) guidelines (6). A combination of terms was used: 'induced membrane'or 'Masquelet' combined with 'bone', and 'rat' (Appendix 1, see section on supplementary materials given at the end of this article). The references of the included studies were also checked to identify other references that should be added. Two authors (MS and FC) did the study selection and data extraction independently. Any disagreement was resolved by discussion between these two authors or by asking a third author for input.

Inclusion and exclusion criteria

Studies were eligible for inclusion if they met the following criteria: *in vivo* rat femoral defect models using biomaterials alone or associated with drug delivery system to improve the IMT. The biomaterials and/or molecules could be either synthetic or natural. Studies were included if the rats underwent circumferential femoral bone defect stabilized by osteosynthesis. The improvement of the IMT could be assessed at the first stage (IM properties) and/or the second stage (bone healing) according to the design of the study. Histologic, radiologic, and/or biomechanical analysis of the IM and/or defect healing was required.

Studies were excluded for the following reasons: language other than French or English, other literature reviews, comments, letters to the editor, book chapters, conference abstracts, no biomaterials used, cadaver study, gene therapy studies, bone defect performed at another localization or implantation in another tissue (subcutaneous, intramuscular), no bone stabilization.

Selection of studies

The articles were initially screened by reading the title and abstract. All the studies that met the eligibility criteria were included in the analysis of whole-text articles retrieved from the databases.

Evaluation of methodological quality

This was done using a checklist based on the ARRIVE 2010 guidelines (Animal Research: Reporting *in vivo* experiments) (7). It evaluates the reliability and reproducibility of *in vivo* animal studies according to 10 criteria (The Essential Set, maximal score: 10/10) considered essential and required for a study of high methodological quality. Eleven additional items (Recommended Set, maximal score: 11/11) were also evaluated to refine the evaluation of the methodology. In this review, the retained studies were evaluated using both sets of the guideline. Each item was scored as 0, 0.5, or 1 (0.5 corresponded to partial validation when certain subitems were missing, maximal score of 21/21). This evaluation was also done independently by two of

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the study authors (MS and FC) and any disagreement was resolved by discussion.

Data extraction

The extracted data pertained to the rat strain, weight, age, number of rats per study, per group, and per analysis methods. Data parameters about the experimental model were: the length of the femoral defect, osteosynthesis methods, and the strategy of the study to improve the IMT. We retrieved details about the groups compared in terms of biomaterials and/or molecules compared, control group parameters, time of induced membrane formation, type of defect grafting, the time before union analysis, and follow-up time. For the histologic analysis, we extracted data about methods and results (histomorphometry, immunohistochemistry, and histologic scores). When a biomechanical assessment had been carried out, we collected data about the methods (compression, bending, traction, etc.) and the results (ultimate failure load, strain, stiffness, and elastic modulus) of analysis. When a radiologic analysis was performed, we retrieved data about the type of imaging, type of analysis of the bone formation and healing of the defect (radiologic scoring, assessment of union, micro-CT analysis). All results are reported at each time-point set by the study.

Results

Selection of studies

A total of 102 studies were identified after removing duplicates. Sixty met one or more of the exclusion criteria and were excluded after screening the title and abstracts. Finally, 20 studies were retained for full-text analysis (Fig. 1).

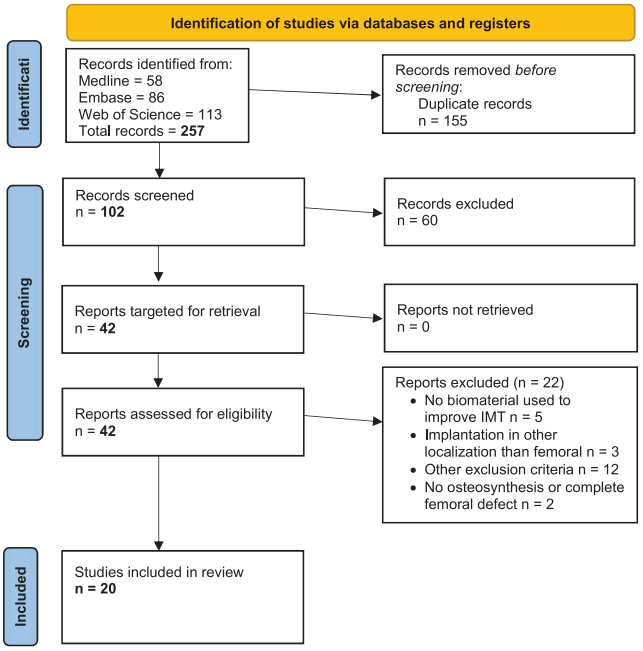

Figure 1

PRISMA flow diagram of this systematic review.

Methodological quality (risk of bias)

The mean score on the ARRIVE Essential 10 checklist was 7.03 ± 0.97 (minimum–maximum 5–8) and the mean total score was 14.6 ± 1.8 (minimum–maximum 11–18) (Supplementary Table 1). The frequently missing items of the Essential Set were the description of the randomization method that was done partially or totally in 13 (65%) studies (8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20) and the blinding assessment of the study's endpoints that were only done in one (5%) study (12). Finally, the *a priori* calculation of the number of animals needed for statistical comparison was only done in 5 (25%) studies (10, 12, 17, 20, 21).

Rat femoral defect models

The most frequent strain was the outbred Sprague-Dawley rat (16/20) (8, 10, 11, 12, 13, 14, 15, 16, 17, 19, 21, 22, 23, 24, 25, 26), followed by Wistar (3/20) (9, 20, 27) and one study used an inbred 344 Fischer strain (18). Only 2/20 studies used female rats (Wistar) (9, 27). The age of the rats mainly ranged from 8 to 12 weeks old. The mean length of the femoral defect was 7 ± 2.2 mm (minimum–maximum 4–10). Stabilization of femoral defect was principally achieved with one plate and 4 screws (13/20) (9, 10, 12, 13, 14, 17, 18, 19, 20, 22, 23, 24, 27) or 6 screws (1/20) (26), followed by external fixation (4/20) (11, 15, 21, 25) and intramedullary nailing (2/20) (8, 16). Two studies used an additional cerclage wiring (9, 26). The defect was grafted in 14/20 studies and consisted of either bone allograft (11, 13, 15, 17, 18, 19, 24), autograft (14, 25) or calcium phosphate material (8, 12, 16, 17, 19, 23, 24, 26) that could be compared. In this model, the time of IM formation was most frequently found at 4 weeks (14/20) (extremes 2–11 weeks), but only three studies analyzed the formation of IM kinetically at several time points (from 2 to 8 weeks) (10, 15, 22) (Supplementary Table 2).

The strategies for improving the induced membrane technique

The main strategy consisted of an optimization of the conventional two-stage IMT (13/20) (8, 10, 11, 14, 15, 17, 18, 19, 20, 21, 24, 25, 27). A part of them (4/13) loaded the PMMA spacer with bioactive molecules such as antibiotics and growth factors (10, 18, 20, 25). One study administered an osteoinductive drug orally (14). Some studies (4/13) focused on replacing the bone autograft at the 2nd stage with a calcium phosphate material

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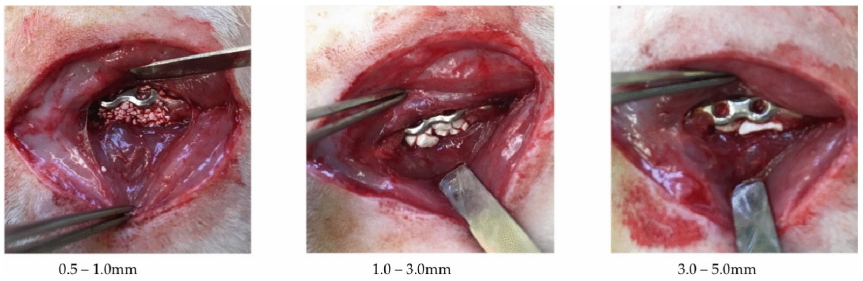

Figure 2

Example of a two-stage strategy replacing bone graft with calcium-phosphate granules (Herafill®) of various size. (©Leiblein *et al.* Impact of scaffold granule size use in Masquelet technique on periosteal reaction: a study in rat femur critical size bone defect model. *European Journal of Trauma and Emergency Surgery* 2022 **48** 679–687. (https://doi.org/10.1007/s00068-020-01516-9)) (No changes were made).

(8, 17, 19, 24) (Fig. 2). The last 4 studies respectively compared a titanium (Ti), silicone or polypropylene spacer (11, 15, 21, 27) to PMMA spacer. Another strategy was to reduce conventional IMT into a single-stage procedure (7/20) (9, 12, 13, 16, 22, 23, 26). Membranes, made of synthetic (9, 23) or natural polymers (16) or derived from human tissue (12, 13, 26), were implanted (Fig. 3). Among them, most studies combined the implantation of a membrane with a calcium phosphate graft, only two studies used single-stage implantation of a calcium sulfate spacer (22) or an artificial membrane alone (9). In both strategies, functionalization could be added to the biomaterial (spacer or membrane), either with BMP7 (8), BMP2 (9, 16, 26), platelet-derived growth factors (16), or bone marrow mononuclear cells (BMMC) (12, 19, 24) (Supplementary Table 2).

Outcomes of the induced membrane technique optimization strategies

Histology

Among the two-stage strategy (13/20), four studies used the PMMA for the first stage but replaced the bone graft in the second stage (8, 17, 19, 24).

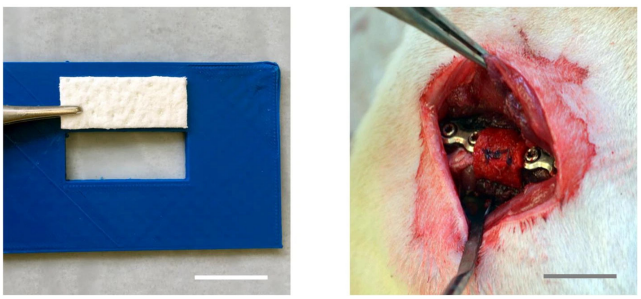

Figure 3

Example of a one-stage procedure using a human acellular dermis (hADM) (Epiflex®) membrane wrapped around a femoral defect, scale bar represents 1 cm. (©Verboket *et al.* From two stages to one: acceleration of the induced membrane (Masquelet) technique using human acellular dermis for the treatment of non-infectious large bone defects. *European Journal of Trauma and Emergency Surgery* 2020 **46** 317–327 (https://doi.org/10.1007/s00068-019-01296-x)) (No changes were made).

These studies focused on the union of the defect. Eight weeks after the second stage, calcium phosphate scaffold material, associated with BMMC (19), mesenchymal stem cells (MSC), and endothelial progenitor cells (EPC) (24) led to an improved osteocalcin staining compared to cancellous bone grafting in one study (19). Filling the defect with calcium phosphate microgranules (0.5–1 mm) improved bone formation compared to cancellous bone grafting in one study (17). But no significant improvement in vascularization was found (19, 24). Four studies functionalized the PMMA to improve the IM properties. At 4 weeks, PMMA + gentamicin improved vascularization or TGFB1 expression vs PMMA + gentamicin and clindamycin or vs a high viscosity PMMA (10, 20). Oral administration of total flavonoid of rhizoma drynariae also showed to improve growth factors expressions of the IM (14). One study performed an injection of PRP or EGF (25) into the bone defect, once a week during 3 weeks between the first and second stage of the IMT. Growth factor expression was higher into the IM and the bone graft compared to no injection of either molecule (26). Finally, 4 studies replaced PMMA with another spacer and focused on analyzing the IM properties. At 4 weeks, compared to PMMA, Ti spacers seemed to improve IM thickness by 35%. In addition, IL-6 expression in the IM was higher when using the Ti spacers with roughened surface compared to those with smooth surface (11). No difference in growth factors expression or cell population and density between PPP and PMMA spacers was found at 4 weeks (15), and no difference between PMMA and silicone spacer found at 11 weeks (27).

In another strategy, five out of seven studies exploring a one-stage procedure provided histologic data, focusing on the union of the defect at a mean time point of 8 weeks. No significant improvement in new bone formation was found between IMT vs a one-stage human amniotic tissue derived membrane functionalized with BMP2 and associated with a calcium phosphate graft (26). However, the combination of a human acellular dermis membrane with a tricalcium phosphate (TCP) graft loaded with BMMC or cancellous bone grafting showed more new bone formation than a two-stage IMT with a PMMA spacer and TCP graft or a cancellous bone graft (12, 13). Functionalization of an artificial PLGA membrane with BMP2 allowed

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a better histologic score than the PLGA membrane alone (9). Calcium sulfate spacer alone did not show higher IM thickness or growth factors expression compared to a PMMA spacer (22). No studies showed histologic improvement of vascularization (12, 22, 26) (Supplementary Table 3).

Biomechanics

Five (25%) studies reported a biomechanical analysis. Gaio *et al.* found that surface-roughened spacers led to more compliant membranes in terms of yielding, strain, elastic modulus, and shrinking capacity, regardless of the type of spacer (PMMA or Ti) (21). One-stage procedures with a human acellular dermis tissue derived membrane, associated with either a TCP graft loaded with BMMC or cancellous allograft were compared to a two-stage procedure with TCP grafting (12) after a PMMA IM or a conventional two-stage IMT (13). Two studies assessed if the cancellous allograft could be replaced with demineralized bone matrix (19) or TCP (24) with or without BMMC, MSC, and EPC loading in a two-stage IMT (19). No difference was found in the femoral three-point bending procedure at 8 weeks for these studies (Supplementary Table 4).

Imaging

Fifteen studies (75%) analyzed union and/or new bone formation with radiographic and/or micro-computed tomography assessment (Supplementary Table 5). Eight studies concerned two stages and reported results from 6 to 12 weeks after the second stage. Replacing a PMMA spacer by a PPP (15) or Ti (11) spacer did not decrease the bone volume or density. Three studies replaced the cancellous graft with calcium phosphate graft in the second stage. Only 1 study reported better union and higher bone volume density with BMP7 functionalization compared with the calcium phosphate material alone (8), whereas the 2 other studies compared the spacer with cancellous bone grafting (19, 24). Three studies compared the effect of adding molecules to the PMMA spacer (tobramycin and vancomycin) (18), or into the defect site (25) (growth factors), or systemically (oral drug administration) (14): two studies reported a radiological improvement of union score or bone mineral density and volume compared to standard IMT (14, 26). Seven studies performed a one-stage procedure, mainly by combining an artificial or human-tissue derived membrane with a calcium phosphate graft (12, 16, 23, 26) that could be functionalized with PDGF and/or BMP2 (16, 26) or BMMC (12). Without functionalization, this combination did not obtain better radiologic scores compared to a two-stage IMT that used a synthetic calcium phosphate filling (23). Functionalizing the synthetic calcium phosphate material could improve the mineralization of the human acellular dermis derived membrane compared to a two-stage IMT at 8 weeks after the one-stage procedure or after the second stage for the control group (12), but did not seem to improve bone union or bone/tissue volume ratio compared to two-stage controls from 2 to 6 weeks after grafting (26). However, an improvement in the radiographic union score was found at later time points for De Baun *et al.* (16). Verboket *et al.* used a cancellous allograft to compare a one-stage procedure with human acellular dermis membrane with two-stage PMMA IMT. No difference in union status and bone mineral density was found at 8 weeks (13). Finally, 2 studies used either an artificial membrane or a calcium sulfate material alone. A PLGA artificial membrane loaded with BMP2 gradually and significantly increased bone formation at 8 weeks compared to no functionalization (9). Using a calcium sulfate material as a spacer in a one-stage procedure seemed to improve bone formation into the defect compared with a PMMA spacer from 4 to 8 weeks post-operatively (22).

Discussion

This systematic review identified two strategies to improve the IMT in the rat femoral defect model, both strategies could be further divided into subcategories basing on various biomaterials, growth factors, or drugs applied, evaluated and compared in the studies.

The rat femoral defect model for studying the improvement of the induced membrane technique

This model was based on quite homogenous parameters. The skeletally mature Sprague-Dawley rat model is an outbred strain that is representative of inter-individual variability when performing an IMT. However, only 5/20 studies performed an *a priori* statistical power and estimation of the number of required subjects (10, 12, 17, 20, 21) and this might explain that several studies found non-significant results. Plate/screw fixation was the most frequent femoral osteosynthesis method. Cortical screws seemed to be the most frequent (6 out of 7 studies), but quite a few studies did not report the type of screw used (10, 14, 17, 18, 19, 27). The mean defect size was 7 mm, which corresponds to an approximately 20% of length of femur, assuming that the median femoral length is 35 mm in this rat strain (28). By definition, a critical-size defect cannot heal by itself and has to exceed 1.5–3.0 times the bone diameter (4). A mature Sprague Dawley femur has a median diameter of 4.5 mm. Thus, a femoral critical-size defect should correspond to 6.75–13.5 mm and only 7/16 (44%) studies that studied bone union had a defect reaching this criterion (11, 12, 16, 17, 22, 23, 24). Moreover, the greatest heterogeneities came from the controls and grafts used in the studies.

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First, several studies used a 'defect left empty' or 'removal of the IM' as their control groups (9, 11, 13, 16, 22, 24, 26). By definition and historical studies in literature, these control groups anyhow would not be able to heal spontaneously. Such controls lead to an increase of animals required in the study. Additionally, the gold standard IMT (to evaluate the induced membrane properties or the defect healing), an unavoidable control when using biomaterials to optimize the IMT, should be performed in each study, but in reality, was used as a control in 14/20 (70%) studies (10, 11, 13, 14, 15, 17, 18, 19, 20, 21, 22, 24, 25, 27). Finally, most studies grafted the defect with allograft bone harvested from donor rats (11, 13, 15, 17, 18, 19, 24). Only two studies used autograft harvested from caudal tail vertebrae (14, 25) Again, using donor rats increases the number of animals required for the studies. The standardization of the above parameters would improve comparability and reproducibility between the studies.

Two-stage procedures to improve the IMT

Thirteen out of 20 studies explored a two-stage procedure and three subcategories were classified: replacing the bone graft with a calcium phosphate material (4/13), replacing the PMMA spacer with another material spacer (4/13), adding a growth factor or an antibiotic to the PMMA spacer, or even a systemic administration of an osteoinductive drug (5/13). Taken together, histological results suggested that calcium-phosphate material, either synthetic or natural, provided better bone formation when used as scaffolds for mesenchymal stems and endothelial progenitor cells. They had consistently lower histological results without functionalization. However, histological results did not corroborate with mechanical or radiological findings (19, 24). Increasing the contact surface of these spacers, for example by using small granules could also improve bone formation (17). Concerning PMMA spacers, the histological properties of the IM seemed to be the best when gentamycin is added to PMMA, notably by increasing the vascularization of the IM (10, 20). This could be further enhanced by locally injection of growth factors, such as PRP or EGF into the defect or by systemic administration of osteoinductive drugs, along with improvement of radiologic bone healing (14, 25). Little to no differences were reported when PMMA was replaced by another spacer. No difference in growth factors expression was reported between PMMA and PPP or Silicone induced membrane, respectively (15, 27). No radiological difference in bone formation was found for Mathieu *et al.* after the 2nd stage (15). For Toth *et al.*, Ti spacers led to a thicker induced membrane than with PMMA. Moreover, roughening the spacer, either in Ti or PMMA, increased IL-6 expression (11). This result could be linked with the results of Leiblein *et al.* who found higher histological bone formation when using calcium phosphate material as small granules, by increasing the surface contact of the spacer (17). Finally, roughening the surface of the spacers could improve the mechanical behavior of the IM by making it more compliant to strain and more elastic (21) (Supplementary Table 6).

One-stage procedures to improve the IMT

Seven studies explored this strategy and focused on the union of the defect. To achieve this goal, an artificial membrane made of synthetic or natural polymer or derived from human tissue was combined with a calcium phosphate filling material. As for two-stage procedures, functionalization with growth factors (BMP2, PDGF) or BMMC was possible. Membranes derived from human acellular dermis provided better histological and radiological bone formation compared to two-stage IMT, either associated with a calcium phosphate graft loaded with BMMC or traditional cancellous bone grafting (12, 13). However, no biomechanical differences were found in three-point bending procedures (12, 13). A BMP2-functionalized calcium phosphate scaffold associated with a membrane derived from human amniotic tissue did not find such results (26). This suggested that loading a spacer with BMMC or using a cancellous bone graft provides higher bone formation than growth factor loading. Studies on polymer-based artificial membranes mostly provided radiological results. Functionalization of these membranes, notably with BMP2, also increased bone volume and union scores compared to the artificial membranes alone (9, 16, 23). However, these studies did not have a two-stage IMT with bone grafting as control group and used a calcium phosphate spacer instead (16, 23), or even no spacer (9) (Supplementary Table 6).

Limitations

This systematic review had some limitations. The analysis of the methodology using the ARRIVE checklist showed that some essential items were missing notably the blinding assessment of the endpoints and the *a priori* calculation of the number of animals required. Moreover, major differences were found in terms of animal models and control groups. The absence of the essential items and the variability of the rat models limit the scope of the conclusions of this systematic review. Finally, this systematic review focused on a single model to identify biomaterials that provided best results in IMT improvement. Other models are studied in this field notably the rabbit model that could bring additional data (29). Other strategies were not included in this work such as genetic studies (30, 31, 32), subcutaneous implantation of biomaterials (33), and other localization of bone defects (34).

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Conclusion

This systematic review of rat femoral defect models using biomaterials to improve the induced membrane technique confirmed that the most common model was a femoral defect of an average length of 7 mm in a skeletally mature Sprague-Dawley rat. The defect was fixed with a plate and four screws. Using a two-stage strategy, the addition of a bioactive molecule to the PMMA spacer (antibiotics), or into the defect site (growth factors) could enhance IM growth factor expression and improve bone formation. Replacing the PMMA spacer with a different spacer resulted in quite similar induced membranes but modulating of the roughness of the spacer roughness could increase their thickness and accelerate their formation. In a one-stage strategy, human-tissue-derived membranes combined with bone grafting achieved bone formation comparable to a standard IMT. Regardless of the strategy used, calcium-phosphate grafts seemed to require a functionalisation with growth factors or bone marrow mononuclear cells to improve outcomes compared with non-functionalised graft. However, the studies included in this systematic review had several methodological limitations that may limit the scope of these conclusions.

Supplementary materials
This is linked to the online version of the paper at https://doi.org/10.1530/EOR-23-0055.

ICMJE Conflict of Interest Statement
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the study reported.

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Author contribution statement
MS: First draft, data extraction, collection and analysis; CZ: writing, revision, corrections; NB: revision, corrections; BM: supervision, revision, corrections; FC: Data extraction, collection, data analysis, extensive revision.

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