Improved cartilage regeneration by implantation of acellular biomaterials after bone marrow stimulation: a systematic review and meta-analysis of animal studies

Michiel W Pot [1] , **Veronica K Gonzales** [2] , **Pieter Buma** [2] , **Joanna IntHout** [3] , **Toin H van Kuppevelt** [1] , **Rob BM de Vries** [4] , **Willeke F Daamen** [Corresp.] [1]

[1] Department of Biochemistry, Radboud Institute for Molecular Life Sciences, Radboud university medical center, Nijmegen, The Netherlands

[2] Department of Orthopedics, Radboud Institute for Molecular Life Sciences, Radboud university medical center, Nijmegen, The Netherlands

[3] Radboud Institute for Health Sciences, Department for Health Evidence, Radboud university medical center, Nijmegen, The Netherlands

[4] SYRCLE (SYstematic Review Centre for Laboratory animal Experimentation), Central Animal Laboratory, Radboud university medical center, Nijmegen, The Netherlands

Corresponding Author: Willeke F Daamen
Email address: Willeke.Daamen@radboudumc.nl

Microfracture surgery may be applied to treat cartilage defects. During the procedure the subchondral bone is penetrated, allowing bone marrow-derived mesenchymal stem cells to migrate towards the defect site and form new cartilage tissue. Microfracture surgery generally results in the formation of mechanically inferior fibrocartilage. As a result, this technique offers only temporary clinical improvement. Tissue engineering and regenerative medicine may improve the outcome of microfracture surgery. Filling the subchondral defect with a biomaterial may provide a template for the formation of new hyaline cartilage tissue. In this study, a systematic review and meta-analysis were performed to assess the current evidence for the efficacy of cartilage regeneration in preclinical models using acellular biomaterials implanted after marrow stimulating techniques (microfracturing and subchondral drilling) compared to the natural healing response of defects. The review aims to provide new insights into the most effective biomaterials, to provide an overview of currently existing knowledge, and to identify potential lacunae in current studies to direct future research. A comprehensive search was systematically performed in PubMed and EMBASE (via OvidSP) using search terms related to tissue engineering, cartilage and animals. Primary studies in which acellular biomaterials were implanted in osteochondral defects in the knee or ankle joint in healthy animals were included and study characteristics tabulated (283 studies out of 6688 studies found). For studies comparing non-treated empty defects to defects containing implanted biomaterials and using semi-quantitative histology as outcome measure, the risk of bias (135 studies) was assessed and outcome data were collected for meta-analysis (151 studies). Random-effects meta-analyses were performed, using cartilage regeneration as

outcome measure on an absolute 0-100% scale. Implantation of acellular biomaterials significantly improved cartilage regeneration by 15.6% compared to non-treated empty defect controls. The addition of biologics to biomaterials significantly improved cartilage regeneration by 7.6% compared to control biomaterials. No significant differences were found between biomaterials from natural or synthetic origin or between scaffolds, hydrogels and blends. No noticeable differences were found in outcome between animal models. The risk of bias assessment indicated poor reporting for the majority of studies, impeding an assessment of the actual risk of bias.

In conclusion, implantation of biomaterials in osteochondral defects improves cartilage regeneration compared to natural healing, which is further improved by the incorporation of biologics.

1 **Improved cartilage regeneration by implantation of acellular biomaterials after**

2 **bone marrow stimulation: a systematic review and meta-analysis of animal**

3 **studies**

4 Michiel W. Pot[1], Veronica K. Gonzales[2], Pieter Buma[2], Joanna IntHout[3], Toin H. van Kuppevelt[1#],

5 Rob B.M. de Vries[4#], Willeke F. Daamen[1*]

6 [1] Department of Biochemistry, Radboud Institute for Molecular Life Sciences, Radboud university

7 medical center, PO Box 9101, 6500 HB Nijmegen, The Netherlands

8 [2] Department of Orthopedics, Radboud Institute for Molecular Life Sciences, Radboud university

9 medical center, PO Box 9101, 6500 HB Nijmegen, The Netherlands

10 [3] Department for Health Evidence, Radboud university medical center, PO Box 9101, 6500 HB

11 Nijmegen, The Netherlands

12 [4] SYRCLE (SYstematic Review Centre for Laboratory animal Experimentation), Central Animal

13 Laboratory, Radboud university medical center, PO Box 9101, 6500 HB Nijmegen, The

14 Netherlands

15 [#] *Contributed equally*

17 *Corresponding author.

18 Willeke Daamen[1]

19 Radboud Institute for Molecular Life Sciences, Radboud university medical center, PO Box 9101,

20 6500 HB Nijmegen, The Netherlands

21 E: Willeke.Daamen@radboudumc.nl, T: +31 24 361 0557, Fax: +31 24 354 0339

Abstract

Microfracture surgery may be applied to treat cartilage defects. During the procedure the subchondral bone is penetrated, allowing bone marrow-derived mesenchymal stem cells to migrate towards the defect site and form new cartilage tissue. Microfracture surgery generally results in the formation of mechanically inferior fibrocartilage. As a result, this technique offers only temporary clinical improvement. Tissue engineering and regenerative medicine may improve the outcome of microfracture surgery. Filling the subchondral defect with a biomaterial may provide a template for the formation of new hyaline cartilage tissue. In this study, a systematic review and meta-analysis were performed to assess the current evidence for the efficacy of cartilage regeneration in preclinical models using acellular biomaterials implanted after marrow stimulating techniques (microfracturing and subchondral drilling) compared to the natural healing response of defects. The review aims to provide new insights into the most effective biomaterials, to provide an overview of currently existing knowledge, and to identify potential lacunae in current studies to direct future research. A comprehensive search was systematically performed in PubMed and EMBASE (via OvidSP) using search terms related to tissue engineering, cartilage and animals. Primary studies in which acellular biomaterials were implanted in osteochondral defects in the knee or ankle joint in healthy animals were included and study characteristics tabulated (283 studies out of 6688 studies found). For studies comparing non-treated empty defects to defects containing implanted biomaterials and using semi-quantitative histology as outcome measure, the risk of bias (135 studies) was assessed and outcome data were collected for meta-analysis (151 studies). Random-effects meta-analyses were performed, using cartilage regeneration as outcome measure on an absolute 0-100% scale. Implantation of acellular biomaterials significantly improved cartilage regeneration by 15.6% compared to non-treated empty defect controls. The addition of biologics to biomaterials significantly improved cartilage regeneration by 7.6% compared to control biomaterials. No significant differences were found between biomaterials from natural or synthetic origin or between scaffolds, hydrogels and blends. No noticeable differences were found in outcome between animal models. The risk of bias assessment indicated poor reporting for the majority of studies, impeding an assessment of the actual risk of bias.

51   In conclusion, implantation of biomaterials in osteochondral defects improves cartilage

52   regeneration compared to natural healing, which is further improved by the incorporation of

53   biologics.

1   Introduction

Articular cartilage is a specialized tissue that covers joint surfaces and provides a low-friction and load-bearing surface for a smooth motion of joints. The structure and function of the tissue can be compromised by traumatic injuries and degenerative joint diseases. Due to its avascular nature, damaged cartilage tissue does not heal spontaneously and it remains a challenge to fully restore tissue function (Ahn et al. 2009; Cao et al. 2012).

The surgical options to treat patients with a localized cartilage defect are limited to cartilage regeneration approaches such as autologous chondrocyte implantation and microfracture surgery (Aulin et al. 2013; Bal et al. 2010). The latter strategy, also known as bone marrow stimulation, is relatively simple, minimally invasive and inexpensive. During this procedure the subchondral bone plate below the cartilage lesion is perforated to initiate bleeding and induce a reparative response. The principle behind this regenerative resurfacing strategy is the migration of non-differentiated bone marrow-derived multipotent stem cells from the subchondral bone into the defect site leading to the formation of new cartilage tissue (Buma et al. 2003; de Mulder et al. 2014; Erggelet et al. 2009). Patients treated with bone marrow stimulation generally show clinical improvements up to 1.5 - 3 years after surgery. However, five years after surgery higher incidences of clinical failures are observed (Hoemann et al. 2010; van der Linden et al. 2013). The newly formed tissue generally consists of fibrocartilage repair tissue rather than hyaline cartilage, has limited filling of the defect, integrates poorly with the surrounding tissue and has inferior mechanical properties compared to hyaline cartilage (Dai et al. 2014). Therefore, the need for regeneration of more durable cartilage tissue persists.

Regenerative medicine and tissue engineering may offer promising alternatives and/or additions to clinical strategies that aim to restore damaged cartilage tissue. The construction of biomaterials and the incorporation of cells and biologics in these implants have been widely investigated for this purpose. Biomaterials can be implanted in osteochondral defects created by applying marrow stimulating techniques (microfracture and subchondral drilling (Falah et al. 2010)) to guide and stimulate the formation of cartilage tissue (Seo et al. 2014). During microfracture surgery an arthroscopic awl is used to penetrate the subchondral bone, while with subchondral drilling a high speed drill is applied to penetrate the trabecular bone. Different strategies have been applied including the implantation of biomaterials with and without cells.

84   Acellular biomaterials offer various advantageous properties such as lack of donor-site

85   morbidity, absence of cell culture costs, off the shelf availability, fewer regulatory issues, and

86   application of one-stage surgical procedures (Brouwer et al. 2011; Efe et al. 2012). Many

87   researchers have explored the approach of implanting acellular biomaterials and investigated

88   the use of various biomaterials *in vivo*, such as natural (*e.g.* collagen (Breinan et al. 2000; Buma

89   et al. 2003; Enea et al. 2013; Wakitani et al. 1994), chitosan (Abarrategi et al. 2010; Bell et al.

90   2013; Guzman-Morales et al. 2014; Hoemann et al. 2007), alginate (Igarashi et al. 2012; Mierisch

91   et al. 2002; Sukegawa et al. 2012) and hyaluronic acid (Aulin et al. 2013; Kayakabe et al. 2006;

92   Marmotti et al. 2012; Solchaga et al. 2000)) and synthetic polymers (*e.g.* polycaprolactone

93   (Christensen et al. 2012; Martinez-Diaz et al. 2010; Mrosek et al. 2010), polyvinyl alcohol

94   (Coburn et al. 2012; Holmes et al. 1975; Krych et al. 2013) and poly(lactic-co-glycolic acid)

95   (Athanasiou et al. 1997; Chang et al. 2012; Cui et al. 2009; Fonseca et al. 2014)). To combine the

96   advantageous properties of these materials, multilayered biomaterials (*e.g.* β-tricalcium

97   phosphate-hydroxyapatite/hyaluronate-atelocollagen (Ahn et al. 2009), ceramic bovine bone-

98   gelatin/gelatin-chondroitin sulfate-sodium hyaluronate (Deng et al. 2012)), blends (*e.g.*

99   poly(glycolic acid)-hyaluronic acid (Erggelet et al. 2009) and type I collagen-hyaluronic acid-

100   fibrinogen hydrogel (Lee et al. 2012)) have been constructed. Biologics are natural factors that

101   can be used to stimulate tissue regeneration, *e.g.* by inducing proliferation and differentiation of

102   cells. Biologics such as growth factors of the transforming growth factor β (TGF-β) superfamily

103   and others have been incorporated in biomaterials to guide and stimulate the formation of

104   hyaline cartilage tissue (Richter 2009). Moreover, it has been reported that the animal model of

105   choice may have a significant impact on study outcome of articular cartilage regeneration

106   (Reinholz et al. 2004). Currently, there is no systematic overview of the current literature

107   assessing the effect of various parameters (*e.g.* applied biomaterials, incorporated biologics and

108   animal models) on cartilage regeneration.

109   The aim of this systematic review and meta-analysis is to assess all current evidence for

110   the efficacy of articular cartilage regeneration using acellular biomaterials implanted in the knee

111   and ankle joint after microfracture and subchondral drilling in animal models. Additionally, we

112   strive to provide transparency on the quality of performed *in vivo* studies, in order to aid the

113   design of future animal experiments and clinical trials. We provide a systematic and unbiased

114   overview of the current literature addressing regeneration of articular cartilage using a wide

115   range of acellular biomaterials containing various biological cues (as illustrated in Fig. 1). Results

116   of semi-quantitative histological scoring systems are used as a quantitative outcome parameter

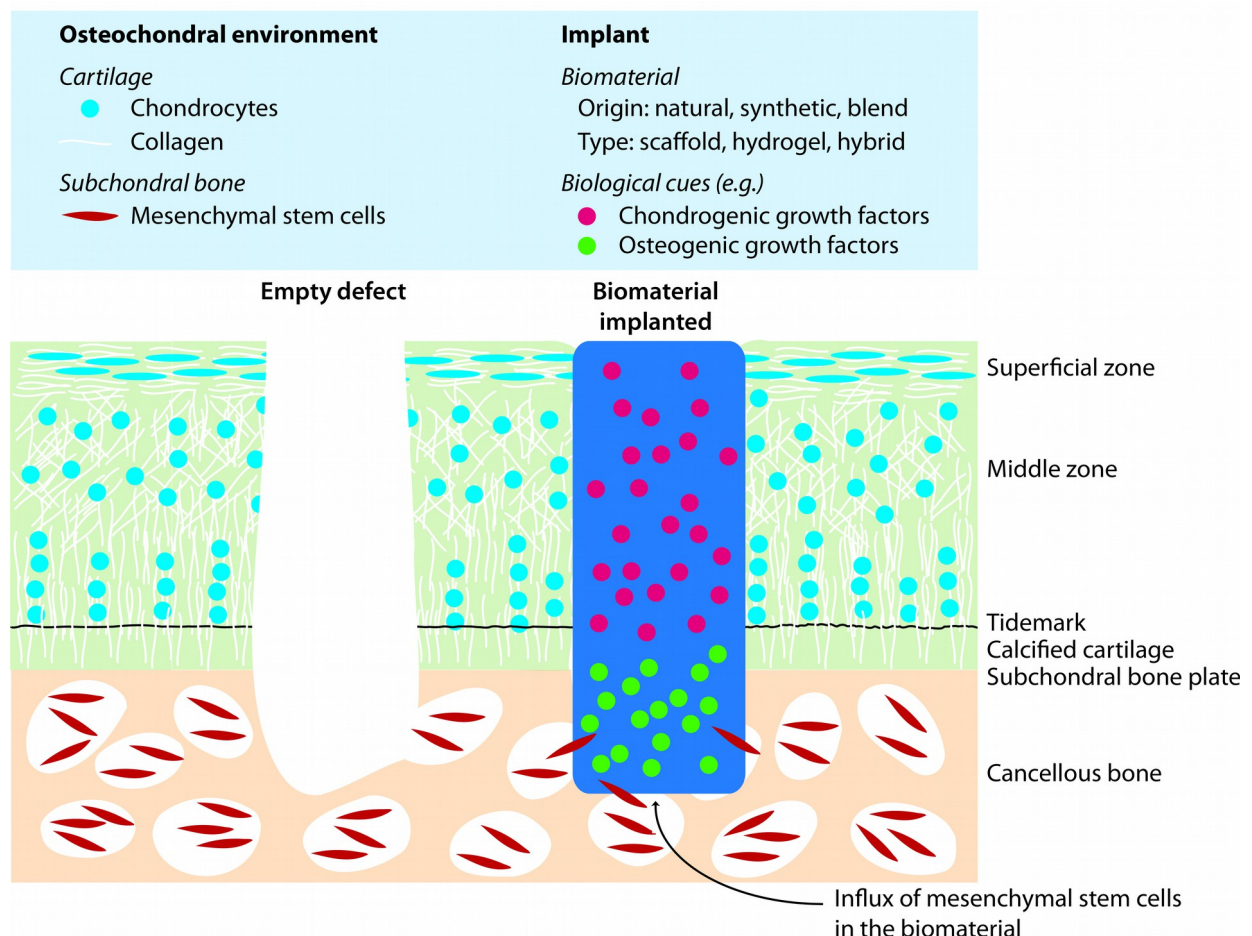
117   for outcome assessment of cartilage regeneration. Although microfracture surgery and

118   subchondral drilling strive to stimulate cartilage and osteochondral regeneration, respectively,

119   both are generalized in this study as cartilage regeneration. Moreover, the evaluation of

120   different subgroups (natural and synthetic origin of the biomaterials, structure of the materials

121   (scaffolds vs. hydrogels), incorporated biological cues, and animal models) was included to gain

122   insights in which parameters affect cartilage regeneration and to what extent.

123   **Figure 1.** Illustration of cartilage regeneration by implantation of biomaterials after bone marrow

124   stimulation. The implanted biomaterials provide a template to guide cartilage regeneration by

125   bone marrow derived mesenchymal stem cells.

2   Materials and Methods

1.1   Search strategy

To identify relevant peer-reviewed articles, a comprehensive search of the literature using PubMed and EMBASE (via OvidSP) was conducted, using the methods defined by de Vries *et al.* *(de Vries et al. 2012)* and Leenaars *et al.* (Leenaars et al. 2012). The last search date was April 3rd 2015. In both databases, a tissue engineering search component developed by Sloff *et al.* *(Sloff et al. 2014)*, consisting of equivalents for tissue engineering (*e.g.* tissue regeneration, regenerative medicine, bio-engineering or biomatrices), was combined with a cartilage search component, consisting of equivalents for cartilage and cartilage-related surgeries (*e.g.* chondral, chondrogenic, surgery, microfracturing or implants). The search components were constructed using MeSH terms (PubMed) and EMTREE terms (EMBASE) and additional free-text words from titles or abstracts ([tiab] or ti,ab). The obtained tissue engineering-related and cartilage-related results were filtered for animal studies using previously described animal search filters (de Vries et al. 2011; Hooijmans et al. 2010). The complete search strategy is attached in Supplementary Information 1. No language restrictions were used.

1.2   Study selection

References from the PubMed and EMBASE search strategies were combined and duplicates were manually removed from EndNote, with the preference of PubMed over EMBASE. All screening phases were performed by two independent reviewers (MP and VG) and reported according to the "Preferred Reporting Items for Systematic Reviews and Meta-Analysis" (PRISMA) guidelines (Higgins 2011). References were first screened based on title and were excluded based on the following criteria: 1) titles showed no relevance to regeneration of articular (hyaline) cartilage, 2) it was specifically stated in the title that the conducted experiment was an *in vitro* study only, 3) osteoarthritis animal models were used, 4) only *ex vivo* studies were performed, and 5) deceased animals were used. In case of doubt or disagreement, references were included for further screening. The second screening phase consisted of a title/abstract screening in Early Review Organizing Software (EROS, Institute of Clinical Effectiveness and Health Policy, Buenos Aires, Argentina, www.eros-systematic-review.org). References were included based on the following inclusion criteria: 1) primary study, 2) animal model, 3) bone marrow stimulation by microfracturing or creation of an osteochondral defect,

156 and 4) biomaterial implantation. Articles were only excluded when it was specifically stated in

157 the abstract that the study was performed without healthy animals or acellular biomaterials, or

158 if biomaterials were not implanted in the knee or ankle joint. Articles were not excluded in case

159 important information in the abstract was missing. These articles were assessed in the full-text

160 screening phase. For the full-text screening, articles were included if they met all of the

161 following inclusion criteria: 1) primary study, 2) animal model, 3) healthy animals, 4) articular

162 cartilage regeneration, 5) knee or ankle joint, 6) bone marrow stimulation by microfracturing or

163 creation of an osteochondral defect, and 7) implantation of an acellular biomaterial. In general,

164 if results of the two reviewers were different, articles were discussed until consensus was

165 reached. In case of double publication, one of the studies was removed. During the screening

166 phase, no selection was made based on publication language. The risk of bias assessment and

167 meta-analysis was applied to studies with a comparison between a non-treated empty defect

168 control and biomaterial implantation, and with semi-quantitative histological scoring system

169 results as outcome data.

1.3   Study characteristics

171 From the studies included after the full-text screening, the following details were obtained:

172 general information (author and year of publication), animal characteristics (species, strain, sex,

173 age, weight and the number of animals), information related to the surgical defect (size, depth

174 and location), experimental conditions, biomaterial, biologics, evaluation time points and all

175 outcome measures used, *i.e.* macroscopic evaluation, semi-quantitative macroscopic evaluation,

176 histology, immunohistochemistry, semi-quantitative histological scoring, and biomechanical

177 tests. Data from semi-quantitative histological scorings were used in the meta-analysis

178 (described in section 2.5). Histological scoring systems applied in different studies consisted of

179 scoring parameters like cell morphology, Safranin-O staining, integrity of surface, thickness,

180 surface of area filled with cells, chondrocyte clustering, degenerative changes, restoration of the

181 subchondral bone and integrity.

1.4   Risk of bias assessment

183 A risk of bias analysis was performed to assess the methodological quality of the studies

184 included in the meta-analysis, using an adapted version of the risk of bias tool described by

185 Hooijmans *et al. (Hooijmans et al. 2014)* (for all included studies containing a 'non-treated

186 empty defect' as control group and studies using semi-quantitative histological scoring systems

187 as outcome measure). A flowchart was constructed (Supplementary Information 2) to score for

188 selection, performance, detection and attrition bias, where the scores '-', '?' and '+' indicate a

189 low, unknown and high risk of bias, respectively. The questions addressed are specified in the

190 Supplementary Information 3. Articles were scored independently by MP and VG, and if the

191 results of the two reviewers were different, results were discussed until consensus was reached.

192 All articles written in Chinese (16 studies) were excluded from the risk of bias assessment only,

193 due to limited resources to independently translate these articles by two native Chinese

194 speakers. However, the data of these studies were extracted and used in the meta-analysis.

195 **1.5   Analysis preparations and meta-analysis**

196 *2.5.1 Analysis preparations*

197 The statistical analyses were restricted to those studies containing the outcome measure semi-

198 quantitative histology, making a comparison between a 'non-treated empty defect' as control

199 group and implanted biomaterials as experimental group. Data (mean, standard deviation (SD)

200 and number of animals) of the control and experimental group were extracted from the studies,

201 for all available time points. When results were not given numerically, but depicted graphically,

202 the mean and SD were measured using ImageJ (1.46r, National Institutes of Health USA). For

203 studies presenting results in boxplots, the mean and standard deviation were recalculated from

204 the median, range and the sample size according the method described by Hozo *et al.* (Hozo et

205 al. 2005). When data were described by a mean and confidence interval (CI), the CI was

206 recalculated   to   a   standard   deviation   by   the   following   equation:

207 $$standard\ deviation = \sqrt{N} \times \frac{up\ per\ limit - lower\ limit}{3.92}$$ for a 95% CI (Higgins 2011). For some

208 studies, data were unclear and assumptions were made, which are listed in Supplementary

209 Information 3a. To compare studies with different histological score system scales, means and

210 standard deviations were converted to a 100% scale by dividing the result by the maximum

211 achievable histological score and multiplying by 100%. In case of missing or unclear data,

212 authors were e-mailed to retrieve the data. When data could not be obtained, these studies

213 were excluded from the meta-analysis (reasons for exclusion are also given in Supplementary

214   Information 2). Results of studies with several experimental groups were combined, following

215   the approach described in the Cochrane Handbook, table 7.7 (Higgins 2011). The same approach

216   was followed to combine results of different animals on several time points in the same group in

217   the same study. One study (Hamanishi, 2013) had an SD of zero, which caused problems in the

218   analyses. Therefore, the SD was changed to 4.29, equal to the SD of the experimental group of

219   the same study at the same time point. The resulting data were used to calculate the treatment

220   effect and corresponding standard error (SE) per study.

221   *2.5.2 Meta-analysis*

222   The following main research question was assessed: Does an overall beneficial effect exist of

223   implanting acellular biomaterials in osteochondral defects compared to non-treated empty

224   defects?

225   First, in order to select the appropriate statistical random-effects meta-analysis model, we

226   compared a univariate approach to the bivariate approach. In the bivariate approach, separate

227   outcomes for control and experimental group were used with their respective SEs. The

228   correlation between these two outcomes was modeled with a compound symmetry covariance

229   matrix, as this resulted in a much lower Akaike Information Criterion value than the use of an

230   unstructured covariance matrix. Results were compared with those of the univariate approach,

231   based on the treatment effect and SE per study. Results of the univariate and bivariate

232   approaches were very similar and we therefore proceeded with the univariate approach, when

233   applicable in combination with likelihood ratio tests.

234   Restricted to the experimental groups, the following sub-questions were addressed to

235   evaluate whether the treatment effect depended on specific variables: 1) Is there a difference

236   between the use of natural and synthetic biomaterials?; 2) Does the structure of the

237   biomaterials affect cartilage regeneration?; 3) Do differences among various material subgroups

238   exist?; 4) Does incorporation of biologics have a beneficial effect on cartilage regeneration

239   compared to control biomaterials?; 5) Do differences among subgroups of biologics exist?; 6) Do

240   different animal models result in variations in cartilage regeneration? Results are shown as %

241   cartilage regeneration (95% CI: [lower CI, upper CI]. Some studies have more than one

242   experimental group. Therefore, the total number of studies and number of experimental groups

243   (no. of studies/groups) are provided.

244   Sensitivity analyses were performed to evaluate the effect of time (*e.g.* all time points,

245   short (<=8 weeks), long time points (>8 weeks), or the maximum time point), outliers (excluding

246   consecutively the studies with the 10% highest / lowest pooled SD, and studies with the 10%

247   highest / lowest SE), implant location, bone marrow stimulating technique applied

248   (microfracturing vs. subchondral drilling), language (excluding studies reported in Chinese as the

249   risk of bias of these studies was not assessed), and excluding studies where assumptions had to

250   be made. Based on a pilot analysis, data of all time points were used for subgroup analyses.

251   Subgroup analyses were only performed for subgroups consisting of more than two groups.

252   The statistical analyses were performed with SAS/STAT® software version 9.2 for

253   Windows, copyright© 2002-2008 by SAS Institute Inc., Cary, NC, USA. The funnel plot shows the

254   overall outcome of the pooled effect size of each study. I^2 was used as a measure of

255   heterogeneity. The forest plot was created with ReviewManager (RevMan, Version 5.3.

256   Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014).

257 **3   Results**

258 **1.6   Search and study inclusion**

259 The searches conducted in PubMed and EMBASE (Supplementary Information 1) resulted in

260 4401 and 5986 studies, respectively, leaving 6688 studies after removal of duplicates. These

261 studies were screened by title and title/abstract, which resulted in 1088 included studies after

262 the title screening and 517 included studies after the title/abstract screening. Screening articles

263 by full-text and subsequently selection for studies with empty defect controls as well as semi-

264 quantitative histology as outcome measure resulted in 283 included studies after full-text

265 assessment, of which 151 and 135 articles could be used for the meta-analysis and risk of bias

266 assessment, respectively (Fig. 2). The studies from Xie *et al. (Xie et al. 2014)*, Yao *et al. (Yao et al.*

267 *2000)* and Zhou *et al. (Zhou & Yu 2014)* could not be retrieved as a full text and these studies

268 were therefore excluded. An overview of all included studies after full-text assessment as well as

269 studies included for the risk of bias assessment and meta-analysis is provided in Supplementary

270 Information 3a. All references and abbreviations can be found in Supplementary Information 3b.

271 In this table, remarks are provided related to exclusion reasons for risk of bias assessment and

272 meta-analysis (*e.g.* duplicate publication and incomplete data). Assumptions made for certain

273 studies are also stated in this table.

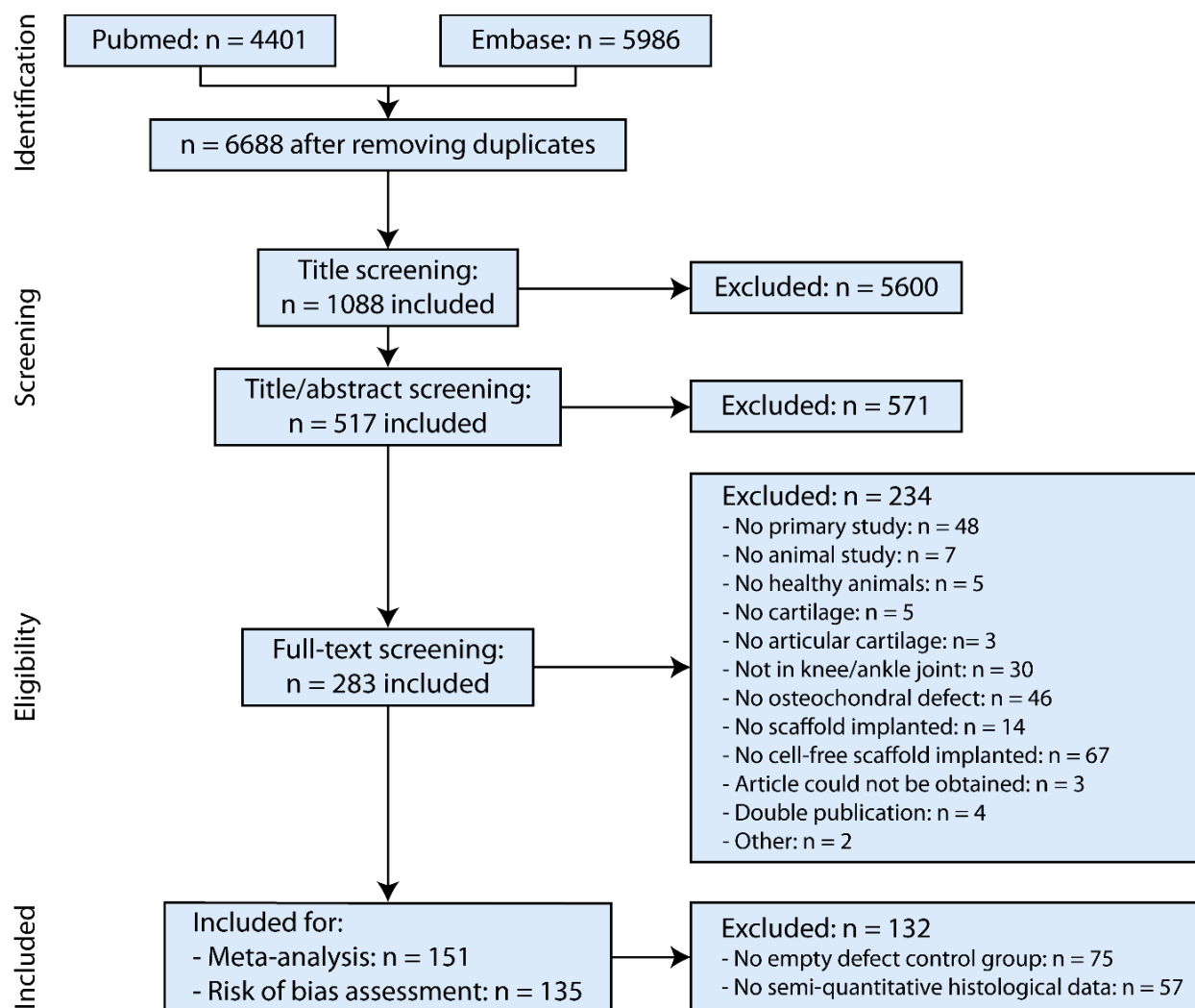



Figure 2. PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analysis) flowchart of the systematic search of literature.

1.7   Study characteristics

The study characteristics (Supplementary Information 3) clearly show substantial variation among studies. A wide range of animal species was used, from small (rat and rabbit) to larger animal models (dog, minipig, goat, pig, sheep and horse). A large variation was observed between the ages of animals (*e.g.* the age of rabbits ranged from 6 weeks to >2 years). Often ages were not described or specified specifically (*e.g.* as adult or mature). Generally, the animals were older (range of years) in large animal models compared to animals used in small animal models (range of months). The defects were created at different locations in the knee joint, such as the trochlea, condyle (medial and lateral), femur and intercondylar fossa. In addition, a large variation was found in the dimensions of the prepared defects, *e.g.* the dimensions of the

286   defects created in rabbits ranged from 4-7 mm in diameter and 0.8-9 mm in depth.

287   Microfracture surgery and subchondral drilling was performed in 25 and 258 studies,

288   respectively. The implanted biomaterials were of natural or synthetic origin or combinations

289   thereof, and consisted of single-layered or multilayered implants or blends thereof. Implants

290   were constructed from a wide range of materials or combinations thereof, such as collagen,

291   chitosan, hyaluronic acid, alginate, fibrin, hydroxyapatite, poly(lactic-co-glycolic acid),

292   polycaprolactone, poly(glycolic acid) and poly(ethylene glycol), and used in different states:

293   scaffolds, hydrogels, or hybrid mixtures of both. Various biological cues were incorporated in the

294   biomaterials prior to implantation or administered afterwards by injection into the knee joint,

295   mostly growth factors of the TGF-β superfamily such as bone morphogenetic protein 2 (BMP-2)

296   and TGF-β1, but also fibroblast growth factor (FGF) and platelet-rich plasma (PRP). The

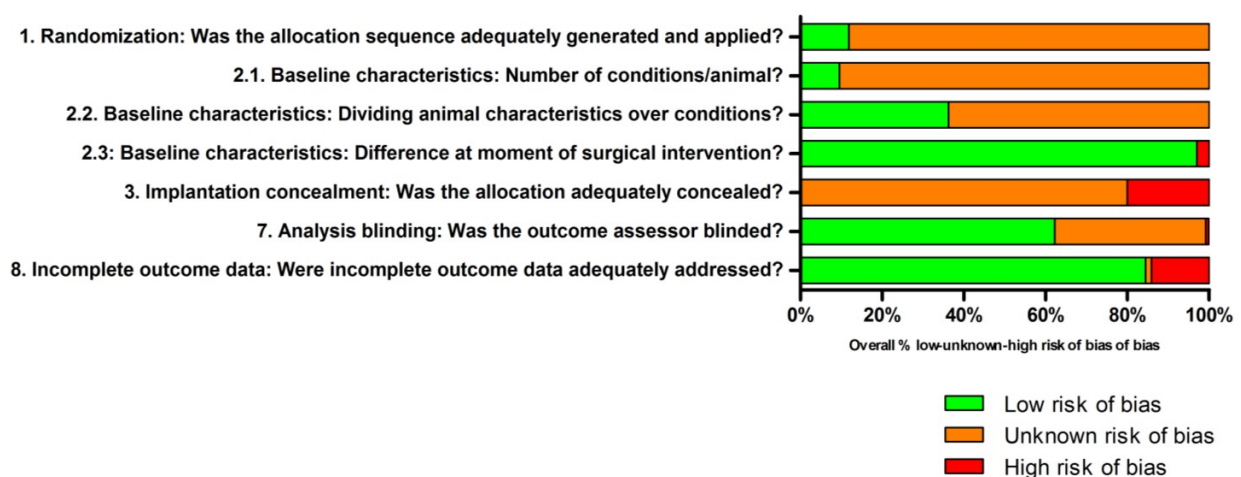
297   maximum follow-up time was 1 year, but studies mainly investigated relatively short-term effects

298   of implanted biomaterials on cartilage regeneration (up to 6 months).

299   **1.8   Risk of bias assessment**

300   A risk of bias assessment was performed to assess risks of bias (selection, performance bias,

301   detection and attrition bias) in studies included for the meta-analysis (Figure 3). An overview of

302   all scores per individual study is provided in Supplementary Information 4.

303   **Figure 3.** Risk of bias of all included studies in the meta-analysis. The green, orange and red

304   colors depict the percentages of studies with low, unknown or high risk of bias of the total

305   number of assessed studies. The risk of bias assessment indicated a general lack of details

306   regarding the experimental setup, as indicated by the orange bars. The green bars represent a

307   low risk of bias, mainly for the difference between groups at the moment of surgical intervention

308   and addressing incomplete outcome data. High risk of bias was infrequently scored, as indicated

309   by the red bars. Q4-Q6 are not depicted in the graph, but are described in Supplementary

310   Information 4.

311   The risk of bias assessment showed that details with respect to the randomization method were

312   not provided (Q1). It was often described that animals were randomized across different groups

313   without describing the method of randomization, thereby limiting assessment of the adequacy

314   of randomization and therefore the actual risk of selection bias. Another notable observation

315   from the experimental designs studied was that only in a limited number of studies it was

316   described that power calculations were performed, whereas sufficient power in animal

317   experiments is a requirement for performing adequate studies. The actual power analyses were

318   never provided in the studies. Due to a lack of information, it was also difficult to assess possible

319   bias by differences in implantation sites (with differences in load-bearing conditions, Q2.1) and

320   differences between groups related to the age, sex and weight of the animals at the start of the

321   experiment (Q2.2). Generally, baseline characteristics of animals prior to implantation of

322   biomaterials (*e.g.* some animals received additional surgery related to harvesting of cells for

323   biomaterials combined with cells, Q2.3) were similar. When implanting biomaterials, no details

324   were described on blinding different biomaterials (Q3). Blinding of the empty defect and

325   biomaterial conditions should be performed to limit bias. However, blinding between the empty

326   defect and biomaterial group is impossible in case only one biomaterial is implanted. More than

327   half of the studies conducted blinded outcome assessment while performing the histological

328   scoring, resulting in low risk of detection bias, whereas the other studies had an unknown risk

329   (Q7). For most studies, no incomplete outcome data were described/found, resulting in low risk

330   of attrition bias. For some studies, dropouts were described/found, resulting in differences

331   between groups and high risk of bias (Q8). Overall, the risk of bias analysis generally revealed

332   poor reporting of the experimental design for the majority of the studies, impeding an

333   assessment of the actual risk of bias.

334 **1.9   Data synthesis**

335   For an overview of the meta-analysis and results obtained, see Table 1. The histological scores of

336   defects implanted with biomaterials and non-treated empty defects are presented as a

337   percentage on a 100% scale, where 0% and 100% indicate poor and perfect cartilage

338   regeneration, respectively. Data are presented as the effect (%) with 95% CI.

Meta-analysis	No. of studies/groups	Subgroups	Cartilage regeneration (% [95% CI])	Mean difference (% [95% CI]) p-value
1. Overall effect	127/400	Biomaterial	53.6 [50.7, 56.6]	15.6 [12.6, 18.6]
	127/247	Empty defect	38.1 [35.1, 41.0]	**p < 0.0001**
2. Origin materials	76/222	Natural	53.0 [49.3, 56.6]	-0.73 [-6.5, 5.0]
	39/137	Synthetic	53.7 [48.8, 58.7]	p = 0.887
3. Material subgroups	20/68	Collagen	49.5 [41.1, 57.8]	
	6/17	Chitosan	57.5 [40.8, 74.2]	
	5/11	Hyaluronic acid	47.9 [31.7, 64.1]	
	5/16	Alginate	63.0 [46.9, 79.00]	
	3/10	Fibrin	55.3 [34.4, 76.3]	p = 0.804
	5/11	Bone	51.2 [35.2, 67.2]	
	15/52	PLGA	58.5 [49.0, 68.0]	
	6/21	PAMPS-PDMAAm DN	47.9 [31.7, 64.1]	
4. Scaffold structure	78/258	Scaffolds	53.1 [49.5, 56.7]	
	41/127	Hydrogels	54.2 [49.4, 59.1]	p = 0.973
	7/17	Blends	55.7 [42.0, 69.3]	
5. Biologicals	113/291	No biologicals	51.7 [48.6, 54.9]	7.56 [2.1, 13.0]
	35/109	Biologicals	59.3 [54.0, 64.6]	**p = 0.007**
6. Biological cues	9/35	BMP	56.6 [-6.3, 119.6]	
	5/20	FGF	51.8 [-43.9, 147.4]	
	8/14	PRP	55.9 [-20.9, 132.8]	p = 0.780
	6/16	TGF	60.2 [-7.5, 128.0]	
7. Animal models	3/5	Dogs	ED: 31.9 [14.5, 49.4]; B: 50.6 [33.0, 68.2]	18.7 [-0.0, 37.3]
	5/13	Goats	ED: 58.5 [43.4, 73.7]; B: 61.6 [47.6, 75.6]	3.1 [-13.2, 19.4]
	1/3	Macaques	ED: 12.2 [-18.2, 42.6]; B: 6.8 [-23.3, 37.0]	-5.4 [-37.6, 26.8]
	10/20	Minipigs	ED: 42.4 [32.4, 52.4]; B: 56.1 [46.3, 66.0]	13.6 [3.1, 24.1]
	94/333	Rabbits	ED: 37.7 [34.2, 41.1]; B: 52.5 [49.0, 55.9]	14.8 [11.1, 18.5]
	13/23	Sheep	ED: 35.3 [26.4, 44.3]; B: 61.3 [52.7, 70.0]	26.0 [16.3, 35.7]
				p = 0.348

339   **Table 1.** Overview of the meta-analysis results for the main research question assessing the

340   overall beneficial effect of implanting acellular biomaterials in osteochondral defects compared

341   to non-treated empty defects and sub-questions evaluating the effect of specific variables on the

342   treatment effect. The total number of studies and number of experimental groups included in

343   the meta-analysis are shown (some studies have >1 experimental group, no. of studies/groups).

344   The quality of cartilage regeneration is presented on a 100% scale, where 100% represents the

345 maximum achievable histological score and thus the best cartilage regeneration. Implantation of

346 biomaterials significantly improved cartilage regeneration compared to non-treated empty

347 defects, which was further improved by the incorporation of biologics. No significant differences

348 were found between natural and synthetic materials, between the various material subgroups,

349 and between the biomaterial structures (hydrogels versus scaffolds versus blends), and between

350 animal species. ED: empty defect, B: biomaterials.

351 *3.1.1   Overall effect biomaterial implantation*

352 The meta-analysis indicates a significant improvement of cartilage regeneration using acellular

353 biomaterials implanted after applying marrow stimulating techniques compared to non-treated

354 empty defects (15.6% (95% CI: [12.6, 18.6], p < 0.0001). The forest plot (Supplementary

355 Information 5) depicts the outcome effect of each individual study. In 73 studies cartilage

356 regeneration significantly improved by the incorporation of biomaterials. In 48 studies no effect

357 was found, whereas in only 6 studies a negative effect on cartilage regeneration was observed. A

358 similar significant effect was observed taking into account the maximum follow-up only (16.3%

359 [13.1, 19.6], p < 0.0001). Also for short and long term follow-up cartilage regeneration was

360 significantly improved (≤ 8 weeks: 12.5% [9.3, 15.7], > 8 weeks: 17.1% [13.9, 20.2]). No notable

361 differences in cartilage regeneration were found between the results based on the maximum

362 follow-up time per study versus those based on all time points per study. Therefore, further

363 subgroup analyses were made using results from all time points together.

364 *3.1.2   Natural and synthetic materials*

365 The subgroup analysis assessing cartilage regeneration using materials of different origin, natural

366 and synthetic, indicated no significant differences (p = 0.887) between natural (53.0% [49.31,

367 56.63]) and synthetic materials (53.7% [48.75, 58.65]).

368   Dividing the group of materials into subgroups allows comparison of cartilage

369 regeneration using different biomaterials. The following subgroups were studied: 1) collagen, 2)

370 chitosan, 3) hyaluronic acid-based biomaterials), 4) alginate, 5) fibrin), 6) bone material-based,

371 7) PLGA, and 8) PAMPS-PDMAAm DN hydrogel. No significant differences between the

372 biomaterial subgroups were found (Table 1).

373   *3.1.3   Material structure*

374   Materials were divided in three groups based on their structure: 1) scaffolds, 2) hydrogels, and

375   3) blends. Cartilage regeneration was similar after use of scaffolds (53.1% [49.53, 56.74]),

376   hydrogels (54.2% [49.39, 59.07]) and blends (55.7% [42.0, 69.3], p = 0.973.

377   *3.1.4   Biologics*

378   Incorporation of biologics in the biomaterials resulted in a statistically significant improvement in

379   cartilage regeneration of 7.6% [2.1, 13.0], p = 0.007, compared to the implantation of control

380   biomaterials. Including only those studies with a direct comparison between control

381   biomaterials and biomaterials loaded with biologics resulted in an improved cartilage

382   regeneration of 14.6% [5.9, 23.4], p = 0.003. Comparing various biological cues including BMP,

383   FGF, PRP and TGF indicated no significant differences in improvement of cartilage regeneration

384   between these biologics.

385   *3.1.5   Animal models*

386   Evaluation of the animal models used showed no significant differences (p = 0.348) between the

387   effects of biomaterials implanted in dogs, goats, macaques, minipigs, pigs, rabbits, rats or sheep

388   (Table 1).

389   *3.1.6   Sensitivity analyses*

390   Sensitivity analyses were performed to assess the robustness of the meta-analysis with respect

391   to the overall effect. The sensitivity analyses indicated that exclusion of studies with

392   assumptions and studies written in Chinese (no risk of bias assessment analyzed) had no effect

393   on the estimated difference in biomaterial regeneration. Moreover, including only studies with

394   SDs or SEs in the 10-90% range did not notably change of the overall outcome effect. In a post-

395   hoc analysis, we investigated cartilage regeneration using biomaterials implanted at different

396   locations including condyles, femur, intercondylar fossa and the trochlea. No differences were

397   found comparing these implant sites (p = 0.143). In another post-hoc analysis, we compared

398   cartilage regeneration of empty defects or defects filled with biomaterials after applying

399   microfracturing or subchondral drilling. For empty defects (p = 0.152) and biomaterial implants

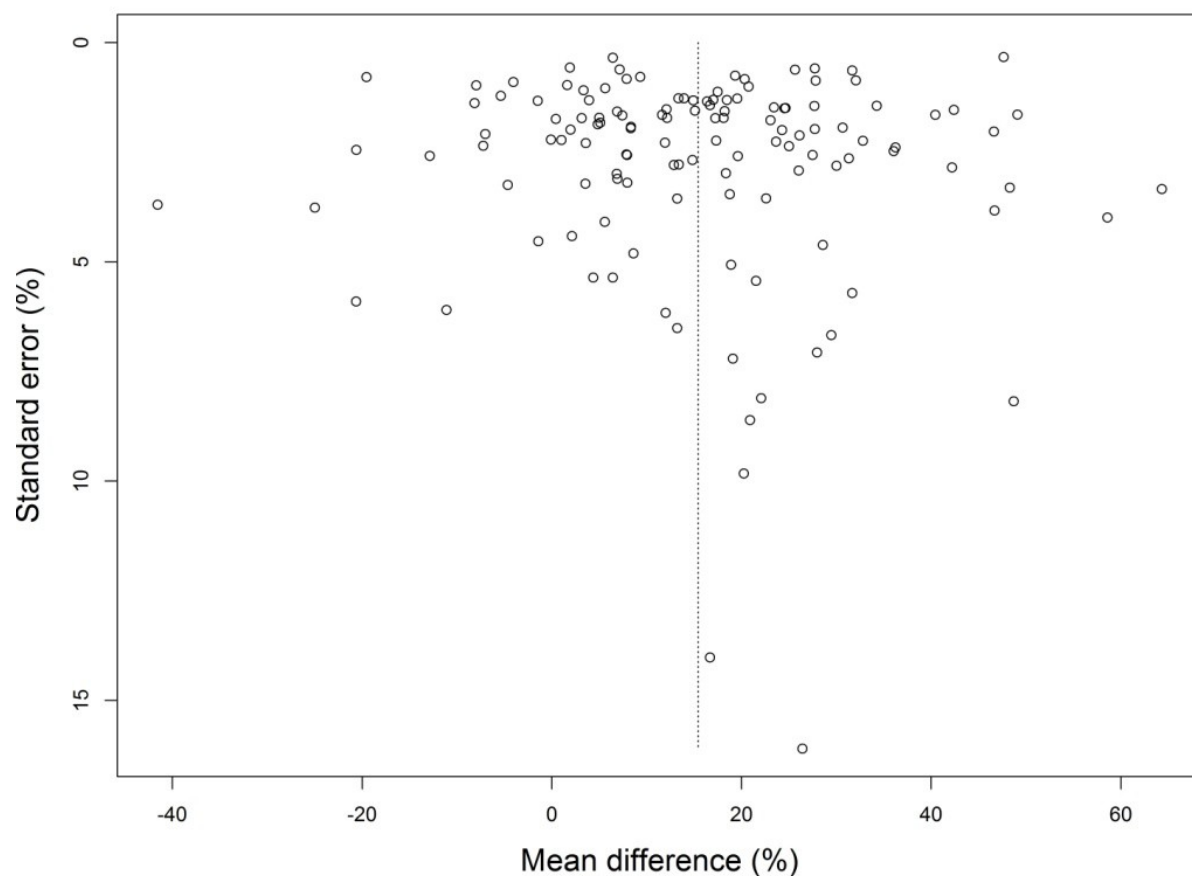
400   (p = 0.063) no significant differences between the two bone marrow stimulating techniques

401   were found.

402 *3.1.7 Publication bias*

403 A funnel plot (Figure 4) was prepared for all included studies to analyze the overall comparison

404 between acellular biomaterials and non-treated empty defect controls. No extensive asymmetry

405 was observed, indicating an absence of considerable publication bias.

406 **Figure 4.** Funnel plot of included studies to assess the overall effect of the implantation of

407 acellular biomaterials compared to non-treated empty defect controls. The figure indicates no

408 substantial asymmetry.

4 Discussion

The regeneration of damaged cartilage has been widely investigated using preclinical models. However, the efficacy of cartilage regeneration using implantation of acellular biomaterials has never been assessed using a systematic review and meta-analysis. This systematic review aimed (a) to provide an overview of currently existing knowledge and identify knowledge gaps, (b) to provide transparency on the quality of performed *in vivo* studies, and (c) to aid the design of future animal studies and clinical trials. The results could provide insight in strategies for future (pre) clinical research related to biomaterial properties, incorporation of biologics, choice of a suitable animal model, and their effects on cartilage regeneration.

The general findings of this systematic review and meta-analysis are that the implantation of biomaterials improves cartilage regeneration compared to non-treated osteochondral defects by 16% (95% CI). There were only six out of 151 studies that showed a negative effect of biomaterial implantation on cartilage regeneration. In 48 studies no significant effect on cartilage regeneration was found. For those studies with improved cartilage regeneration (73 studies), clinical studies will have to confirm the beneficial effect of implantation of biomaterials on cartilage regeneration in human patients. Filardo *et al.* described the implantation of an osteochondral biomimetic scaffold consisting of a type I collagen cartilage-like layer, a type I collagen/hydroxyapatite intermediate layer, and a mineralized blend of type I collagen and hydroxyapatite as a subchondral bone compartment, to treat patients with osteochondritis dissecans. For these patients, clinical scores improved significantly after the first two years and evaluation by MRI indicated good defect filling and implant integration, but also heterogeneous tissue regeneration and changes of the subchondral bone (Filardo et al. 2013). In two studies included in this systematic review and meta-analysis, this osteochondral biomimetic scaffold was also implanted in sheep. Cartilage regeneration after six months was 81.8% ± 8.9% (empty defect: 23.2% ± 20.7%) and 81.2% ± 5.1% (empty defect: 23.4% ± 6.7%). A direct comparison between the degree of cartilage regeneration described in the preclinical studies and clinical study is not possible since no histological results were described in the clinical study. In addition, outcome measures used in preclinical studies may not predict the clinical outcome. For example, a randomized controlled clinical trial with BST-CarGel, a chitosan-based medical device, showed greater lesion filling and superior repair tissue quality compared to bone marrow stimulation after twelve months implantation, but without notable clinical differences related to pain,

440 stiffness and physical function between both groups (Stanish et al. 2013). A remarkable
441 observation is the difference in follow-up between the studies, which may explain the good
442 histological scores in the preclinical studies after six months and heterogeneous tissue
443 regeneration and changes of the subchondral bone after two years in human patients. In
444 general, clinical studies demonstrated improved cartilage regeneration by the implantation of
445 biomaterials after bone marrow stimulation, but there is still room for improvement regarding
446 clinical outcome and tissue quality.

447   The only subgroup analysis that showed a statistically significant result between the
448 groups was between control biomaterials and biomaterials loaded with biologics. In future
449 clinical studies assessment of the beneficial properties of implanting biomaterials loaded with
450 biologics is of interest, since a significant improvement of 8% (95% CI) compared to control
451 biomaterials was found and even 14.6% when using studies that directly compared biomaterials
452 with and without biologics. We were not able to perform analyses for the effect of the
453 concentration or subtype of the growth factors due to the small size of these subgroups,
454 although these factors may have a large effect on the outcome. In the study by Ishii *et al.* a
455 positive effect of FGF-2 was observed by the addition of at least 183 ng to the biomaterials (Ishii
456 et al. 2007), while Maehara *et al.* showed significant improvements of impregnating of
457 biomaterials in 10 µg/ml and not for 100 µg/ml FGF-2 (Maehara et al. 2010). Loading
458 biomaterials with different BMPs including BMP-2 (Aulin et al. 2013; Reyes et al. 2012; Reyes et
459 al. 2014; Reyes et al. 2013; Tamai et al. 2005) and BMP-7 (Mori et al. 2013), or TGF subtypes
460 including TGF-β (Mierisch et al. 2002) and TGF-β1 (Reyes et al. 2012; Reyes et al. 2014), resulted
461 in significantly improved cartilage regeneration. However, for clinical application of these
462 medical devices, one should take safety of the products into account as side effects of TGF-β in a
463 joint environment, including fibrosis and osteophyte formation, have been described (Blaney
464 Davidson et al. 2007) and patients suffered from major complications after spinal surgery and
465 implantation of high concentrations of BMP/INFUSE (Epstein 2013).

466   The study characteristics of all included studies were tabulated to provide an extensive
467 overview of the available literature. Besides the internal validity of the studies, the
468 generalizability (external validity) of the study results is of great importance. The latter is
469 affected by factors related to the animal model (species, strain, weight, age, and sex), surgery
470 (location and size of the defect) and follow-up, resulting in heterogeneity between studies. This

471   was also indicated by the relatively high level of heterogeneity (I^2) for the main meta-analysis

472   (99.4% [99.4, 99.4]), and the heterogeneity was almost similar for subgroup analyses. We chose

473   to include only healthy animals receiving biomaterials. The screened studies also contained

474   osteoarthritis models that were not included, which may be relevant for future applications to

475   treat patients with osteoarthritis. Therefore, results from this systematic review and meta-

476   analysis may be different compared to results found for osteoarthritis models and future clinical

477   studies with osteoarthritis patients. We assumed that in order to assess the effect of implanted

478   biomaterials on cartilage regeneration, reduction of the influence of confounding parameters

479   would aid the validity of the results and conclusions. In this study, the meta-analysis included all

480   available data of the effect of implanting biomaterials after applying bone marrow stimulating

481   techniques (microfracture and subchondral drilling) compared to empty defects on cartilage

482   regeneration. During microfracture surgery the subchondral bone is penetrated using an

483   arthroscopic awl, whereas during subchondral drilling the trabecular bone is penetrated using a

484   high speed drill, which may result in thermal necrosis (Falah et al. 2010). Remarkably, more

485   studies applied subchondral drilling (258 studies) compared to microfracture surgery (25

486   studies), while microfracture surgery was developed to overcome problems associated with

487   thermal necrosis from subchondral drilling in the treatment of human patients (Kane et al.

488   2013). We did perform a post-hoc meta-analysis to investigate differences in cartilage

489   regeneration after applying both marrow stimulating techniques and subsequent implantation

490   of biomaterials, which resulted in no significant differences between microfracturing and

491   subchondral drilling. A reason for the larger number of animal studies performing subchondral

492   drilling compared to microfracture surgery may be the ease to perform subchondral drilling over

493   microfracture surgery in animals. Although in the included studies, various implant locations (*i.e.*

494   trochlea and condyles) were used, we grouped the results in the meta-analysis. A post-hoc

495   subgroup analysis was performed to compare defect locations, but no overall significant

496   differences were found for biomaterials implanted at different implant locations. Our analysis

497   did not confirm a finding of Chen *et al.* showing improved chondrogenesis in trochlear versus

498   condylar cartilage defects after bone marrow stimulation in rabbits (Chen et al. 2013). This may

499   be explained by various parameters affecting the degree of cartilage regeneration at different

500   implant locations, such as the animal model, follow-up period and rehabilitation protocol.

501   Different outcome measures such as macroscopic and histological evaluation, semi-
502 quantitative macroscopical and histological evaluation using scoring systems, histomorphometry,
503 PCR and biochemical assays were used to assess the regenerative potential of implanting
504 biomaterials. In this systematic review and meta-analysis, only data from semi-quantitative
505 histological scoring systems were used as outcome measure. We chose to use these data as
506 most authors presented their results by this method and it allows quantitative comparison of
507 different studies in a meta-analysis. Various histological scoring systems have been used by the
508 authors of included studies, such as the O'Driscoll, Pineda, Wakitani and ICRS scoring system,
509 which were also reviewed by Rutgers *et al.* (Rutgers et al. 2010). Depending on the histological
510 scoring system, parameters such as cell morphology, matrix staining, surface regularity,
511 structural integrity, defect filling and the restoration of the subchondral bone were evaluated. A
512 limitation of this outcome measure is that the specific topics addressed in the scoring systems
513 greatly differ, *i.e.* some studies focus on the regeneration of cartilage only, cartilage as well as
514 subchondral bone, or include a biomaterial component (*e.g.* scoring degradation of the implant).
515 Other outcome measures including macroscopic evaluation, biochemical analysis and
516 biomechanical aspects of the tissue may complete the overview of the tissue quality and provide
517 valuable insights in articular cartilage regeneration, but these outcome measures were only used
518 in a limited number of studies, and therefore not assessed in this analysis.

519   The risk of bias assessment provided insights in the quality of the experimental design of
520 the studies. Most studies scored a low or unknown risk of bias, however, also little high risk of
521 bias was scored. Low methodological quality (internal validity) may result in an overestimation
522 or underestimation of the intervention effect (Higgins et al. 2011). In general, details regarding
523 the randomization procedure were not described. Moreover, an observation during the risk of
524 bias assessment was that only few studies included in the systematic review described that
525 power calculations were performed, which is a crucial aspect in conducting experimental studies
526 to ensure sufficient power of experimental designs. As a consequence, studies may lack
527 sufficient power and thereby run the risk of false negative results. Due to the poor reporting of
528 the experimental design for the majority of the studies the assessment of the adequacy of
529 randomization and power calculations, and thus the assessment of the actual risk of selection
530 bias, was inadequate. However, it may also hold true that studies were well designed but there
531 was only poor reporting of the experimental designs (Hooijmans et al. 2012). Most researchers

532 scoring the histology sections were blinded and sections were randomized. However, when

533 biomaterials are not (completely) degraded, blinding between biomaterials and empty defects is

534 practically impossible. A lack of blinding of outcome assessors implies the risk of

535 detection/observer bias (Bello et al. 2014). Bias may have been introduced by the lack of

536 blinding and randomization and detracts from the overall validity of the results (Bebarta et al.

537 2003; Hirst et al. 2014). There is a risk that the positive results found are an overestimation of

538 the true effect of using biomaterials. Introducing standardized protocols such as the golden

539 standard publication checklist (Hooijmans et al. 2011) or the ARRIVE guidelines (Kilkenny et al.

540 2012) may improve reporting of animal studies.

541 Funnel plots represent the precision of the measured effects, which increases by an

542 increase in study size. Therefore, for small and large studies scatter will be relatively large and

543 little, respectively. As a consequence, generally, in the absence of bias the plot resembles a

544 symmetrical pyramid (a funnel) (Higgins 2011). An important limitation may be publication bias,

545 since multiple studies were included from the same author and negative results may not be

546 published. It was described in a study by ter Riet *et al.* that researchers themselves estimate that

547 only 50% of the conducted animal experiments are published. This problem may be solved by

548 statistical corrections for publication bias (ter Riet et al. 2012). In our study, the funnel plot did

549 not show asymmetry and therefore did not indicate the presence of publication bias.

550 The translational value of animal studies depends on the comparability to the clinical

551 situation. One of the limitations of the performed animal experiments is the short follow-up

552 times. The maximum follow-up time was one year, but most studies investigated cartilage

553 regeneration up to 6 months. This limits the translational value since clinical improvements in

554 humans are generally observed up to 1.5 - 3 years after microfracture surgery (Hoemann et al.

555 2010; van der Linden et al. 2013). Moreover, many variations were present in the applied animal

556 models, *i.e.* animal characteristics (species, strain, sex, age, weight), surgical defects (size, depth

557 and location), applied biomaterials, and incorporated biologics. A review by Chu *et al.* (*Chu et al.*

558 *2010)* extensively reflects on benefits and limitations of different animal models used in cartilage

559 repair studies. They state that for humans the volume of a cartilage defect is approximately 550

560 mm^3 and treatment is required for defects with a surface larger than 10 mm^2. Due to the limited

561 joint size of many animals, larger animal models such as minipig, goat and horse therefore offer

562 superior translational value than smaller animals such as rats, rabbits and dogs. However, all

563   studies contained defect volumes smaller than 550 mm^3 and only few studies had defects
564   surfaces larger than 10 mm^2. Additionally, cartilage thickness differs among various species, with
565   goat, rabbit, minipig and dogs having thinner cartilage than humans. Another drawback for
566   some animal models is the large endogenous repair potential. In humans, untreated defects
567   show little to no regeneration while rabbits display a large regenerative potential, limiting clinical
568   translation. Dog, goat, minipig and horse do not have this large endogenous repair and the use
569   of these animals may therefore be favorable. The maturity of the animals is of great importance
570   when designing animal experiments since open growth plates can impede with the applied
571   treatment. Animal species are skeletally mature at different ages; *i.e.* rabbits at the age of 16-39
572   weeks, pigs at 42-52 weeks, dogs at 12-24 months, sheep and goat at 24-36 months and horses
573   at 60-72 months (Ahern et al. 2009; Chu et al. 2010). In this study we did not group studies
574   based on animal maturity. In addition to clinical relevance, other reasons to select an animal
575   model are related to logistical, financial, and ethical considerations. A systematic review
576   conducted by Ahern *et al.* (Ahern et al. 2009) investigated the strengths and shortcomings of
577   different animal models and compared these with common clinical lesions in clinical studies.
578   They remarked that smaller animal models are often used due to feasibility, while large animal
579   models may more closely resemble humans. However, no differences were found between
580   animal models in this systematic review and meta-analysis, which may be explained by various
581   parameters affecting the degree of cartilage regeneration such as implant location, defect size,
582   follow-up period and rehabilitation protocol.

583   In this systematic review and meta-analysis the efficacy of cartilage regeneration using
584   acellular biomaterials was compared to the natural healing response of defect treated with
585   microfracture surgery and subchondral drilling. The risk of bias assessment indicated poor
586   reporting in animal studies, which may be improved in future animal studies. Moreover, to
587   improve the translation towards clinical trials animal experiments should be comparable to the
588   clinical situation. As described in this systematic review a relatively high level of heterogeneity
589   exists between studies related to the animal model, surgery and follow-up, with a need to
590   resemble current clinical settings more closely. In this study we only addressed bone marrow
591   stimulating techniques (microfracture and subchondral drilling) and subsequently the
592   incorporation of biomaterials, but also the regeneration of partial thickness cartilage defects
593   may be beneficial to prevent progression to full-thickness cartilage defects, limit the progression

594   towards osteoarthritis and improve quality of life in patients. In many studies also cell-laden

595   biomaterials have been implanted and the beneficial effect of cellular biomaterials versus

596   acellular biomaterials and the natural healing response has been studied. Although acellular

597   biomaterials offer various advantageous properties over cellular biomaterials such as no donor-

598   site, no cell culture, off the shelf availability, less regulatory issues, and application of one-stage

599   surgical procedures (Brouwer et al. 2011; Efe et al. 2012), studying the additive value of cellular

600   biomaterials may aid further improvement of marrow stimulating techniques.

601 **5   Conclusion**

602 The systematic review and meta-analysis resulted in a structured, thorough and transparent

603 overview of literature related to the current evidence for the efficacy of cartilage regeneration

604 using acellular biomaterials implanted after microfracturing in animal models. Cartilage

605 regeneration is more effective by implantation of acellular biomaterials in microfracture defects

606 compared to microfracturing alone. The efficacy is further improved by the incorporation of

607 biologics.

608 **6   Manuscript information and declarations**

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