Biomaterials Science

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *Biomater. Sci.*, 2020, **8**, 4792

Bioinorganic supplementation of calcium phosphate-based bone substitutes to improve *in vivo* performance: a systematic review and meta-analysis of animal studies†

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Supplementation of CaP-based bone graft substitutes with bioinorganics such as strontium, zinc or silicon is an interesting approach to increase the biological performance in terms of bone regenerative potential of calcium phosphate (CaP)-based bone substitutes. However, the *in vivo* efficacy of this approach has not been systematically analyzed, yet. Consequently, we performed a systematic review using the available literature regarding the effect of bioinorganic supplementation in CaP-based biomaterials on new bone formation and material degradation in preclinical animal bone defect models and studied this effect quantitatively by performing a meta-analysis. Additional subgroup analyses were used to study the effect of different bioinorganics, animal model, or phase category of CaP-based biomaterial on bone formation or material degradation. Results show that bioinorganic supplementation increases new bone formation (standardized mean difference [SMD]: 1.43 SD, confidence interval [CI]: 1.13–1.73). Additional subgroup analysis showed that strontium, magnesium and silica significantly enhanced bone formation, while zinc did not have any effect. This effect of bioinorganic supplementation on new bone formation was stronger for DCPD or β-TCP and biphasic CaPs than for HA or α-TCP ($p < 0.001$). In general, material degradation was slightly hindered by bioinorganic supplementation (mean difference [MD]: 0.84%, CI: 0.01–1.66), with the exception of strontium that significantly enhanced degradation. Overall, bioinorganic supplementation represents an effective approach to enhance the biological performance of CaP-based bone substitutes.

Received 15th April 2020,
Accepted 21st July 2020

DOI: 10.1039/d0bm00599a

rsc.li/biomaterials-science

1. Introduction

Bone is one of the most commonly transplanted tissues with over 2 million bone graft procedures performed annually worldwide.[1] However, bone regeneration is still a challenge for large defects or in patients with systemic diseases that negatively affect bone regeneration (*e.g.*, osteoporosis[2] or diabetes mellitus[3]). Autografts, *i.e.* a patient's own bone, is the gold standard in treating bone defects resulting from *e.g.* trauma or tumor removal. However, this therapeutic approach shows many disadvantages, including low availability, additional surgical site for tissue harvest, and donor site morbidity.[4] In order to overcome these problems, research has focused on synthetic bone grafts, which are off-the-shelf available and do not require a second surgical site. Amongst available synthetic alternatives for bone graft substitutes, calcium phosphate (CaP)-based bone substitutes are preferred due to their similarity in crystalline structure and chemistry to the inorganic phase of natural bone, which offers them excellent biocompatibility.[5,6] Depending on the Ca/P molar ratio, different CaP compounds can be distinguished, which have different physicochemical properties (Table 1). CaP-based bone substitutes are applied as bone substitute material in the form of granules, blocks or cements.

The inorganic portion of bone primarily includes calcium phosphates with hydroxyapatite as the most abundant phase,

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/d0bm00599a

Table 1 Main CaP compounds used as bone substitutes and their Ca/P ratio[7–9]

Compound and typical abbreviation	Chemical formula	Ca/P ratio
Dicalcium phosphate anhydrous (DCPA)	$CaHPO_4$	1
Dicalcium phosphate dihydrate (DCPD)	$CaHPO_4{\cdot}H_2O$	1
Amorphous calcium phosphate (ACP)	$Ca_xH_y(PO_4)_z{\cdot}nH_2O$, n = 3–4.5; 15–20% H_2O	1.2–2.2
α-Tricalcium phosphate (α-TCP)	α-$Ca_3(PO_4)_2$	1.5
β-Tricalcium phosphate (β-TCP)	β-$Ca_3(PO_4)_2$	1.5
Calcium deficient hydroxyapatite (CDHA)	$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$	1.5–1.67
Hydroxyapatite (HA)	$Ca_{10}(PO_4)_6(OH)_2$	1.67
Tetracalcium phosphate (TTCP)	$CaO{\cdot}Ca_3(PO_4)_2$	2.0

and trace amounts of bioinorganics such as magnesium, zinc, strontium or fluorine.[10–12] Therefore, addition of bioinorganics to synthetic CaP-based bone substitutes is an interesting approach to improve their resemblance with the inorganic composition of bone and to potentially enhance the biological properties (*e.g.* increase bone regeneration potential). Multiple bioinorganics have been added to CaPs with variable biological effects. Among them, strontium, silicon, magnesium and zinc seem to be the most studied ones, and have been supplemented to CaPs mainly either by substitution of one atom by the other (*e.g.* Sr for Ca) or by physical entrapment in the lattice. Strontium (Sr) is an element chemically similar to calcium, and can substitute calcium ions in osteoblast-mediated processes.[13] Multiple studies have shown that Sr has a beneficial effect on bone tissue by enhancing osteoblast activity, while inhibiting osteoclast-mediated resorption.[14–16] Silicon (Si) plays an essential role in bone formation, mineralization and crosslinking of collagen and proteoglycans during bone growth.[17–19] For example, the presence of Si in bioactive glasses, another widely used synthetic bone graft substitute, was described to benefit initial cell adherence, which in turn increases cell proliferation and differentiation.[20] Although different studies have shown that Si-supplementation of CaP-based bone substitutes enhanced osteoblast proliferation and differentiation,[21,22] others showed contradictory results.[23] Another effect of Si is, that when supplemented to CaP ceramics, it enhances their stability.[24,25] Magnesium (Mg) is one of the most abundant ions in the human body with >50% of the total Mg amount present within bone tissue. Mg improves bone metabolism by enhancing cell proliferation and differentiation and maintaining the normal function of parathyroid glands and metabolism of vitamin D.[26,27] Mg deficiency is a risk factor of osteoporosis.[28–31] *In vivo* studies have shown that Mg-supplementation in CaP-based bone substitutes implanted in the maxillary sinus floor enhanced their biodegradation and improved their osteoconductivity compared to Mg-free controls.[32] Zinc (Zn) is required for the growth, development and maintenance of healthy bones, in which it stimulates osteoblast activity, inhibits osteoclast's resorptive function, and enhances bone protein synthesis, leading to increased bone mass and growth.[33,34] Cultures of osteoblasts or osteoblast precursors in the presence of Zn ions have shown an upregulation of osteogenic marker gene expression, while Zn deficiency downregulated their expression.[35–37] In combination with CaP-based bone substitutes, Zn has shown to enhance osteoblast proliferation *in vitro* and enhance osteoconduction and osteoinduction *in vivo*.[38–42]

In general, bioinorganic supplementation to CaP-based bone substitutes has been largely studied and many studies have explored the effect of bioinorganic supplementation on the physico-chemical characteristics and *in vitro* and *in vivo* behavior of the CaP-based bone substitutes. Specifically, mainly small animal models (*i.e.* rat or rabbit) have been considered, although some studies have included larger animals, such as sheep or dogs. However, the use in human patients has been limited and only CaPs with Mg[43–45] and Si[46–48] have been implanted. To further enhance their clinical applicability it is important to review the current available data of preclinical studies in order to set a direction for future preclinical and clinical studies. Further, in view of the ambiguous effects of bioinorganic supplementation of CaP-based bone substitutes, there is a clear need to create an overview of all relevant *in vivo* studies. To this end, *in vivo* studies using experimental animal bone defect models were retrieved from the literature to analyze the effect of bioinorganic supplementation of CaP-based bone substitutes on bone formation and material degradation. Studies including synthetic CaP-based bone substitutes supplemented with any bioinorganic were analyzed. For comparison reasons, it was important that the CaP-based bone substitute without the bioinorganic was also one of the studied groups and that only one bioinorganic was studied. To this end, the available literature was systematically reviewed for data on this topic and gathered data was used to perform a meta-analysis, where the main outcome measures were new bone formation (NBF) and remaining material (RM), both collected from histomorphometric data.

2. Methods

2.1. Search strategy

The study protocol was designed following SYRCLE (SYstematic Review Center for Laboratory animal Experimentation) guidelines (ESI Fig. S1†).[49] For identification of all original papers on the topic, we systematically searched PubMed and Embase (*via* OvidSP). The search was conducted on the 27th November 2018 without any language restrictions and consisted of four main components: "bone regeneration",

"bone substitutes", "bioinorganics" and "animal studies". For each component, relevant thesaurus terms were collected and synonyms were identified for application in a title/abstract search [TiAb]. The full search strategies in PubMed and Embase are depicted in Tables S1 and S2,† respectively.

2.2. Paper selection

The selection process was carried out using SyRF (CAMARADES, UK) and divided into two phases. In the first phase, two reviewers (E-C. G., and J. B.) independently performed the paper selection based on title and abstract. Differences were resolved by a third reviewer (I. L. T.). In the second phase, the full text of the selected papers of the first phase was reviewed by two independent reviewers (I. L. T. and J. B). Differences were resolved by discussion until mutual agreement was reached. The inclusion and exclusion criteria for these two phases are depicted in Table S3.†

2.3. Data extraction and quality assessment

The study characteristics data were extracted by I. L. T. from each selected paper: animal species, strain, number of defects per group and time point, category of CaP-based biomaterial, bioinorganic supplement, number of relevant groups, surgical site, implantation period and main outcomes are presented in Table 2. This Table 2 shows a summary of all the study characteristics extracted (Table S4†). Bibliographic details (*e.g.* author, year of publication, and language), animal numbers/characteristics/medical condition, dose of bioinorganic and number of defects per animal and their size were also registered (Table S4†).

Additionally, (histo-)morphometric data on bone formation and/or material degradation were extracted for meta-analysis. For all included papers, outcome data for experimental and control groups were extracted if mean, standard deviation (SD) or standard error (SE), and number of defects per group (n) were reported or could be recalculated. If (histo-)morphometric data were presented only graphically, data were remeasured using image analysis software (Fiji 1.51n, ImageJ, National Institutes of Health, Bethesda, MD, USA).[50]

The risk of bias was assessed using SYRCLE's risk of bias tool.[51] Each paper was subjected to 9 questions related to the general risk of bias and 3 questions related to quality of reporting of study quality items. The risk of bias was categorized as low, unclear, or high, the reporting quality as yes or no. Two independent reviewers (I. L. T. and R. K. G.) performed quality assessment of all included papers. Disagreements were resolved by discussion until mutual agreement was reached.

2.4. Data synthesis and statistical analysis

Data were meta-analyzed using Review Manager Version 5.3.2 (Copenhagen, The Nordic Cochrane Centre, The Cochrane Collaboration, 2012). Forest plots were used to display individual and overall effect sizes. New bone formation (NBF) and remaining material (RM) data were extracted from the included papers and standardized mean differences (SMD; for NBF) or mean differences (MD; for RM) and 95% confidence intervals (CIs) were calculated per study/comparison. Overall effect sizes were computed using a random effects model. Heterogeneity was assessed using I^2. When a paper included measurements at different implantation periods, the outcome was extracted at every time point. Exceptionally, when the same animals were used for measurements at all implantation periods (*i.e.* without sacrificing the animals at each time point), only the measurements of the sacrificial time point were considered. When a control group was used as comparison for different experimental groups, the number of defects in the control group was divided by the number of experimental groups to avoid multiple comparisons with the same defects.

To explore possible causes of heterogeneity and to assess the influence of several variables, subgroup analyses (*post hoc* analyses) were performed on the condition that three or more independent papers with five or more comparisons were available. The variables included in the subgroup analyses were 'type of bioinorganic', 'animal species' and 'category of CaP-based bone substitute'.

3. Results

3.1. Paper identification and selection

The systematic literature search identified 1341 references in PubMed and 1094 references in Embase, leading to a total of 1943 references after removal of duplicates (Fig. 1). Of those, 1768 references were excluded in the title and abstract screening phase, leaving 175 papers for full-text evaluation. After full-text study, 99 papers were excluded based on exclusion criteria (Fig. 1) and 76 papers were included in the systematic review. Some papers were excluded for more than one exclusion criterion. One paper was not analyzed in the further steps of the review because it was in Japanese and there were no resources that allowed proper translation,[122] one paper was retracted[123] and three papers were excluded because they used non-synthetic CaPs.[124–126] In the meta-analysis, 45 papers with 133 quantitative bone formation comparisons and 20 studies with 57 quantitative remaining material comparisons could be included.

3.2. Description of characteristic of included papers

The main characteristics of the included papers are listed in Table 2, ranked in alphabetical order of first author's surname. Further details of the characteristics (*e.g.* animal numbers/characteristics/medical condition, dose of bioinorganic and number of defects per animal and their size) are provided in Table S4.†

The included papers showed use of various animal species, including rabbits (40 papers), rats (27 papers), sheep (5 papers), dogs (2 papers), goats (1 paper) and pigs (1 paper), with predominantly healthy animals (65 papers, ~85%). In 11 papers, animals (female) were subjected to bilateral ovariectomy-induced osteoporosis and in one paper animals were subjected to steroid-induced osteonecrosis. Bone defects were created in different sites, including femur (33 papers), tibia

Table 2 Characteristics of the papers included in the systematic review. Papers where the outcome measure was only histology was not included in the meta-analysis

Paper ID	Species-strain (disease)	Material	Type of bioinorganic	Number of groups (relevant for SR)	Defects/group/time point	Surgical site	Implantation period	Outcome
Aparicio *et al.* (2016)[52]	Rabbit-NZ	Ground CPC (brushite)	Si	6 (5)	3	Calvaria	8 weeks	NBF (%); RM (%)
Baier *et al.* (2013)[53]	Rat-SDW (osteoporotic)	CPC (apatite)	Sr	2	10[c]	Femur	1,3 and 6 months	NBF (%)
Bose *et al.* (2018)[54]	Rat-SDW	β-TCP	Fe	3 (2)	NA	Femur	4, 8 and 12 weeks	NBF (%)[a]
Bunpetch *et al.* (2018)[55]	Rabbit-NZ	β-TCP	Si	3 (2)	7	Femur	8 and 16 weeks	NBF (%)
Cabrejos-Azama *et al.* (2014)[26]	Rabbit-NZ	Crushed CPC (brushite)	Mg	4	3,5	Calvaria	8 weeks	NBF (%); RM (%)[a]
Calasans-Maia *et al.* (2014)[56]	Rabbit-NZ	HA cylinders	Zn	2	10	Tibia	1, 2 and 4 weeks	NBF (%)[a]
Calvo-Guirado *et al.* (2015)[57]	Rabbit-NZ	HA and β-TCP granules (4Bone)	Si	3 (2)	7	Calvaria	8 and 12 weeks	NBF (%); RM (%)
Camiré *et al.* (2006)[58]	Rabbit-HL	CPC (apatite)	Si	2	6[d]	Tibia	1, 2 and 3 weeks	Histology [b]
Cardemil *et al.* (2013)[59]	Rat-SDW (healthy and osteoporotic)	HA granules	Sr	4	8	Femur	6 days and 4 weeks	NBF (%); RM (%)[a]
Carmo *et al.* (2018)[60]	Rat-W	Carbonated HA	Sr	2	5	Maxilla	1 and 6	NBF (%); RM (%)
Chandran *et al.* (2016)[61]	Rat-W (osteoporotic)	HA granules	Sr	3 (2)	6	Femur	8 weeks	Regeneration efficiency ratio[a]
Chandran *et al.* (2018)[62]	Sheep (osteoporotic)	HA	Sr	4 (2)	2	Femur	2 months	Regeneration efficiency ratio
Cheng *et al.* (2014)[63]	Rat-SDW (osteoporotic)	CPC	Sr	3 (2)	7	Femur	6 weeks	Histology [b]
Chissov *et al.* (2008)[64]	Rat-W	HA granules	Si	6 (2)	3	Tibia	3, 6, 9, 12 weeks and 6 and 9 months	Histology [b]
Cho *et al.* (2014)[65]	Rabbit-NZ	HA disks	Cl	2	8	Calvaria	4 weeks	NBF (%); implanted granules (%)
Chou *et al.* (2013)[66]	Rat-W	β-TCP	Zn	3 (2)	10[d]	Tibia	0, 4 and 8 weeks	BMD (mg cm^{-3})
Costa *et al.* (2016)[67]	Rat-W	β-TCP granules	Mg	6 (2)	6	Calvaria	1 and 6 months	NBF (%); RM (%); connective tissue (%)
Dagang *et al.* (2008)[68]	Rabbit-NZ	CPC (apatite)	Sr	4 (3)	1	Femur	4, 8, 12 and 24 weeks	Resorption degree[a]
Deng *et al.* (2017)[69]	Goat-NA	Ha	Mg	3 (2)	4	Calvaria	4, 8, 12 weeks	NBF (%)
Elgali *et al.* (2016)[70]	Rat-SDW	HA granules	Sr	3 (2)	6	Femur	12 h, 3 and 6 days	NBF (%)
Gong *et al.* (2016)[71]	Rabbit-NZ	CPC	Si	3	14	Tibia	8 and 12 weeks	NBF and RM[a]
Gu *et al.* (2001)[72]	Rabbit-NZ	HA blocks	Sr	4 (3)	4	Mandible	1, 3 and 6 months	NBF (%)[a]
Gu *et al.* (2013)[73]	Rabbit-NZ	CPP	Sr	6 (2)	4	Radius	4, 8 and 16 weeks	NBF/RM ratio[a]
Guo *et al.* (2018)[74]	Rat-SDW (osteoporotic)	Nano-HA particles	Sr	2	10	Femur	4 and 8 weeks	NBF (%)
Hing *et al.* (2006)[75]	Rabbit-NZ	HA cylinders	Si	5	4	Femur	1, 3, 6 and 12 weeks	Normalized NBF (%)
Inoue *et al.* (2005)[76]	Rat-W	CDA	F	4	2	Tibia	1 and 2 weeks	NBF (%)
Inoue *et al.* (2011)[77]	Rat-W	CDA	F	4	2	Tibia	1 and 2 weeks	Histology [b]
Kamitakahara *et al.* (2016)[78]	Rat-W	α-TCP granules	Si	2	3	Femur	4, 8, 12 and 24 weeks	NBF (%); RM (%)
Kang *et al.* (2015)[79]	Rabbit-JW (osteonecrotic)	CPP	Sr	3 (2)	6	Femur	4, 8 and 12 weeks	NBF[a]
Kawamura *et al.* (2000)[40]	Rabbit-NZ	TCP and TCP/HA ceramics	Zn	8	6	Femur	4 weeks	NBF (%)
Kawamura *et al.* (2003)[41]	Rabbit-NZ	TCP/HA ceramic	Zn	2	6	Femur	2, 4, 6, 12, 24 and 60 weeks	NBF (%)
Kaygili *et al.* (2015)[80]	Rabbit-NZ	HA	Sr	7 (6)	28	Tibia	4 weeks	NBF[a]
Ke *et al.* (2017)[81]	Rabbit-NZ	β-TCP microspheres	Si	4 (3)	8	Femur	6, 12 and 18 weeks	NBF (%); RM (%)
Kuang *et al.* (2015)[82]	Rat-SDW	CPC	Sr	2	5	Femur	32 weeks	NBF (%); RM (%)[a]
Laufer *et al.* (1988)[83]	Dog-NA	CaP cylinders	F	4 (2)	5	Femur	8 months	Histology [b]
Li *et al.* (2009)[84]	Rabbit-NZ	CPC (apatite)	Zn	2	6	Tibia	4 weeks	NBF (%)

Table 2 *(Contd.)*

Paper ID	Species-strain (disease)	Material	Type of bioinorganic	Number of groups (relevant for SR)	Defects/group/ time point	Surgical site	Implantation period	Outcome
Li *et al.* (2016a)[85]	Rat-SDW (osteoporotic)	HA	Sr	2	13	Tibia	8 weeks	NBF (%)
Li *et al.* (2016b)[86]	Rat-SDW	CPC	Li	2	10	Tibia	1 and 2 months	NBF (%)
Li *et al.* (2018)[87]	Rabbit-JW	CPP	Cu	4 (2)	5	Tibia	4 and 8 weeks	NBF (%)
Liao *et al.* (2002)[88]	Rabbit-NZ	HA	Sr	4 (3)	4	Mandible	1, 3 and 6 months	Histology [b]
Liu *et al.* (2013)[89]	Rabbit-NZ	β-TCP	Si	4 (3)	6	Femur	4, 12 and 26 weeks	NBF (%); degradation rate (%)
Luo *et al.* (2018)[90]	Rabbit-NZ	HA	Sr	3 (2)	NA	Calvaria	4, 8 and 12 weeks	NBF (%)[a]
Machado *et al.* (2016)[91]	Sheep-Santa Ines	HA microspheres	Sr	3 (2)	5	Tibia	30 days	NBF (%); RM (%)
Masaeli *et al.* (2016)[92]	Rat-SDW	CPC (brushite)	Sr	3 (2)	10	Calvaria	4 weeks	Histology [b]
Maté-Sánchez de Val *et al.* (2012)[93]	Rabbit-NZ	HA block	Si	3	20	Tibia	60 days	NBF (%)
Mohan *et al.* (2013)[94]	Rabbit-NZ	HA	Sr	2	3	Ulna	4 and 12 weeks	NBF (%); RM (%)
Mueller *et al.* (2017)[95]	Rat-SDW	CPP	Sr	3 (2)	NA	Calvaria	8 and 12 weeks	Histology [b]
Patel *et al.* (2002)[96]	Rabbit-NZ	HA granules	Si	2	NA	Femur	23 days	NBF (%)[a]
Patel *et al.* (2005)[97]	Sheep-TC	HA granules	Si	3	4	Femur	6 and 12 weeks	NBF (%)
Pina *et al.* (2010)[98]	Pig-NA	CPC (brushite)	Zn	3 (2)	1	Tarsal bone	1 month	NBF[a]
Porter *et al.* (2003)[99]	Sheep-TC	HA granules	Si	2	NA	Femur	6 and 12 weeks	Histology [b]
Preethanath *et al.* (2016)[100]	Rabbit-NZ	HA granules	Si	4 (2)	8	Femur	8 weeks	NBF (%)
Reitmaier *et al.* (2018)[101]	Sheep-Merino	CPC (apatite)	Sr	2	7	Femur and tibia	6 and 26 weeks	NBF (%); RM (%)
Rentsch *et al.* (2018)[102]	Rat-W	CPC (brushite)	Cr	3	10	Tibia	3 and 6 months	NBF (%); RM (%)
Resende *et al.* (2013)[103]	Rabbit-NZ	HA spheres	Zn	2	5	Tibia	26, 52 and 78 weeks	RM (%)
Roh *et al.* (2016)[104]	Rat-SDW	MBCP™	Si	6 (2)	6[c]	Calvaria	4 and 8 weeks	BMD (mg/cm3)
Salamanna *et al.* (2019)[105]	Rat-SDW (osteoporotic)	HA nanocrystals	Sr	10 (6)	10[c]	Vertebra	8 weeks	NBF (%)[a]
Schendel *et al.* (2009)[106]	Rabbit-NZ	CPC (apatite)	Mg	3 (2)	NA	Calvaria	2, 12 and 24 weeks	NBF[a]
Suruagy *et al.* (2016)[107]	Rabbit-NZ	HA discs	Zn	2	5	Calvaria	12 weeks	NBF (%); RM (%)
Tao *et al.* (2018)[108]	Rat-SDW (osteoporotic)	CPC	Sr	4 (2)	5	Femur	8 weeks	NBF (%); RM (%)
Thormann *et al.* (2013)[109]	Rat-SDW (osteoporotic)	CPC (apatite)	Sr	3 (2)	15	Femur	6 weeks	NBF (%)
Tian *et al.* (2009)[110]	Rabbit-NZ	CPP	Sr	2	8	Radius	4, 8 and 16 weeks	NBF (%); RM (%)
Tripathi *et al.* (2018)[111]	Rabbit-JW	β-TCP	Mg	2	4	Femur	4, 12 and 24 weeks	RM (%)
Vahabzadeh *et al.* (2015)[112]	Rat-SDW	CPC (brushite)	Si	4	3	Femur	4, 8 and 12 weeks	NBF (%)
Valiense *et al.* (2016)[16]	Rabbit-NZ	Carbonated HA granules	Sr	2	6	Maxilla	4 and 12 weeks	NBF (%); RM (%)
Velasquez *et al.* (2013)[113]	Rabbit-NZ	α-TCP	Si	4 (3)	5	Tibia	2, 4 and 8 weeks	RM (%)[a]
Vestermark *et al.* (2011)[114]	Dog-AF	HA granules	Sr	2	10	Humerus	4 weeks	NBF (%)
Wang *et al.* (2012)[115]	Rabbit-NZ	β-TCP scaffold	Si	4 (3)	5	Femur	4, 12 and 26 weeks	NBF (%), degradation rate (%)
Wei *et al.* (2010)[116]	Rabbit-NZ	CPC (apatite)	Mg	4	6	Femur	1, 2, 3 and 6 months	NBF (%)

Table 2 (Contd.)

Paper ID	Species-strain (disease)	Material	Type of bioinorganic	Number of groups (relevant for SR)	Defects/group/time point	Surgical site	Implantation period	Outcome
Wu *et al.* (2008)[117]	Rabbit-NZ	CPC (apatite)	Mg	2	3	Femur	1, 2, 3 and 6 months	NBF (%)
Xu *et al.* (2008)[118]	Rabbit-NZ	β-TCP disks	Si	2	4	Calvaria	4, 8 and 16 weeks	NBF (%); RM (%)
Yassuda *et al.* (2013)[119]	Rat-W	β-TCP granules	Mg	3 (2)	5	Maxilla	1, 3 and 6 weeks	NBF (mm^3); RM (mm^3)[a]
Yu *et al.* (2017)[120]	Rat-SDW	ACP porous microspheres	Sr	3 (2)	12	Calvaria	8 weeks	NBF (%)
Zarins *et al.* (2018)[167]	Rabbit-Ca (osteoporotic)	HA/TCP	Sr	4 (2)	7	Femur	12 weeks	Histology[b]
Zeng *et al.* (2012)[32]	Rabbit-NZ	CPC (brushite)	Mg	6 (2–3)	6	Maxilla	2 and 8 weeks	NBF (%); RM (%)
Zhang *et al.* (2013)[121]	Rabbit-NZ	CPC (brushite)	S	4 (2)	6	Femur	8 weeks	NBF (%)

AF: American foxhund; Ca: Californian; HL: Holland lop; JW: Japanese white; NZ: New Zealand; SDW: Spargue-Dawley; TC: texcel × continental; W: wistar; α/β-TCP: alpha/beta-tricalcium phosphate; ACP: amorphous calcium phosphate; DCPA: dicalcium phosphate anhydrous; CaP: calcium phosphate; CDA: calcium deficient apatite; CPC: calcium phosphate cement; CPP: calcium polyphosphate; HA: hydroxyapatite; TTCP: tetracalcium phosphate; PCL: poly(ε-caprolactone); NBF: new bone formation; RM: remaining material; BMD: bone mineral density. [a] Quantitative data available but not included in meta-analysis (boxplots or ratios, no SD or no sample number). [b] Only qualitative data available. [c] Not all groups same sample number for analysis. [d] Same animals were used for all timepoints; NA: not available.

(17 papers), calvaria (14 papers), maxilla (4 papers), mandible (2 papers), radius (2 papers), humerus (1 paper), ulna (1 paper), vertebra (1 paper) and tarsal bone (1 paper). Regarding the CaP-based materials used, 44 papers used slow degrading ceramics (HA or α-TCP, from now on HA/α-TCP), 18 papers used fast degrading ceramics (DCPD or β-TCP, from now on DCPD/β-TCP), 5 papers used a combination of HA and β-TCP or a combination of TTCP and DCPA (from now on, biphasic CaPs) and 9 used other CaPs, including calcium polyphosphates (CPPs) or calcium phosphate cements (CPCs) with unknown exact composition. In addition, a wide range of bioinorganics were used; strontium (Sr, 31 papers), silicon (Si, 20 papers), magnesium (Mg, 9 papers), zinc (Zn, 8 papers), fluoride (F, 3 papers), chloride (Cl, 1 paper), chromium (Cr, 1 paper), copper (Cu, 1 paper), iron (Fe, 1 paper), lithium (Li, 1 paper) and sulfur (S, 1 paper). A graphical overview of these categories can be observed in Fig. S2.† In all papers that involved the use of diseased animals (either osteoporosis or osteonecrosis, 12 papers), CaP-based bone substitutes were supplemented with Sr. Further, the bioinorganics dose, defect size and implantation time widely varied among studies. Regarding publication date, Fig. 2 shows an increase in number of papers particularly over the last ten years. Trends in the use of bioinorganics are also clear: while Si was the most common bioinorganic used between 2000 and 2013, Sr became the most commonly used bioinorganic for supplementation after 2013.

3.3. Risk of bias and quality of included studies

Fig. 3 presents the overall results of the risk of bias and reporting quality assessment of the 76 papers included in the systematic review. Overall, the reporting of quality items was poor. Randomization was mentioned in less than 50% of the included papers, blinding was seldom mentioned and only 3 papers addressed how the sample size was calculated. Additionally, only 5.3% of the included papers indicated the randomization method followed and this method was considered adequate and in as few as 2.6% of the papers the implant allocation was correctly concealed. In addition, 22.4% of the comprised papers registered low risk of bias regarding to performance bias items "random housing" and "blinding of the caregivers" and 26.3% were scored as low risk of bias for the detection bias item "random outcome assessment". For this detection bias item, it is important to mention that even more than 70% of the studies showed an 'unclear' risk of bias, because bone formation is a rather slow process, the exact order of sacrifice is not critical. As a rule, studies where each animal had more than one group implanted, a low risk of bias was considered for "random housing" and "blinding of the caregivers" and "random outcome assessment". Finally, 11.8% of the papers scored high risk of bias for the attrition bias item "incomplete outcome data" and 18.4% of the papers appeared to have other problems, mainly, an unclear number of animals or defects per animal or a low number of animals/time point/group. Although most studies showed poor reporting which led to unclear risk of bias, none of the papers was excluded based on its quality or risk of bias assessment.

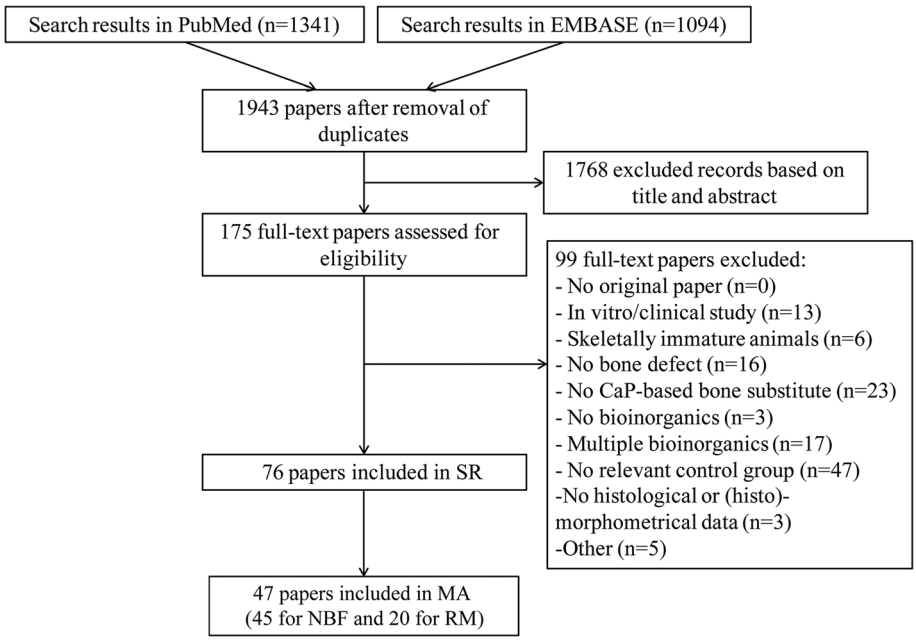

Fig. 1 Flow diagram showing literature search and selection results. SR: systematic review; MA: meta-analysis; NBF: new bone formation; RM: remaining material.

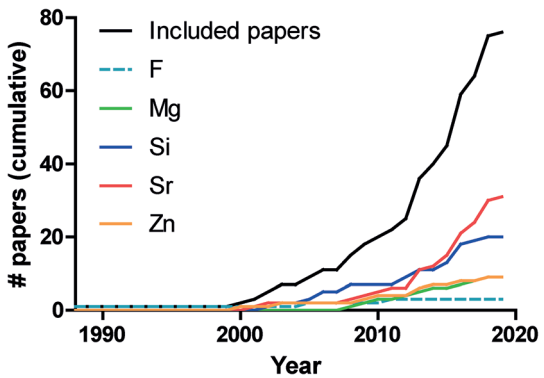

Fig. 2 Cumulative number of papers published per year that were included in the systematic review, as a whole, and depending on the type of bioinorganic used. After a single paper in 1988, the number of papers increased after 2000. Bioinorganics that were used in only one paper were not included (*i.e.* Cl, Cr, Cu, Fe, Li and S).

3.4. Meta-analysis of outcome measures

New bone formation (NBF) and remaining material (RM) were the outcome measures included in the meta-analysis (Fig. 4). Additional subgroup analyses were conducted for variables that likely influence bone formation and material degradation, *i.e.* type of bioinorganic, animal species, and category of CaP-based bone substitute (HA/α-TCP, DCPD/β-TCP, or biphasic CaP). To ensure reliability, subgroup analysis was only performed if at least five different comparisons from at least three different papers were available.

New bone formation. Forty-five papers with 133 bone formation comparisons met the inclusion criteria for meta-analysis of NBF (expressed as number of SDs difference; hereafter: SD) (Table 3 & Fig. S3†). The analysis contained 134 experimental groups, including data of 1345 bone defects. The overall effect of bioinorganics on new bone formation (*i.e.* SMD intra-paper comparisons of CaP-based bone substitute supplemented with a bioinorganic *vs.* control CaP-based bone substitute) was 1.43 SD with 95% CI = [1.13, 1.73] and a relatively high heterogeneity of 71%. In 52 comparisons, the supplementation of bioinorganics significantly increased NBF, while only one comparison showed that bioinorganic supplementation significantly decreased NBF. Regarding the type of bioinorganic, supplementation with Mg, Si or Sr significantly increased NBF, while Zn showed to have no effect. Incorporation of Zn had significantly less effect on NBF than incorporation of any of the other bioinorganics ($p < 0.001$). Bioinorganic supplementation significantly enhanced NBF in all animal species and increased NBF regardless of the type of CaP-based bone substitute, but was particularly prominent for DCPD/β-TCP and biphasic CaPs ($p < 0.001$). In general, subgroup analysis decreased the heterogeneity, but this slight decrease does not explain the heterogeneity.

Remaining material. Twenty papers with 57 remaining material comparisons met the inclusion criteria for meta-analysis of RM (%) (Table 4 & Fig. S4†). The analysis contained 57 experimental groups, including data of 616 bone defects. The overall effect of bioinorganic supplementation on RM (*i.e.* MD between CaP-based bone substitute and CaP-based bone substitute with bioinorganics) was 0.84% with 95% CI = [0.01, 1.66] and a very high heterogeneity of 100%. For interpretation of the results, it is important to understand that a 'positive' effect (MD > 0) means that there was more RM in bioinorganic supplemented materials than in control materials without bioinorganic. In 22 comparisons, bioinorganic supplementation

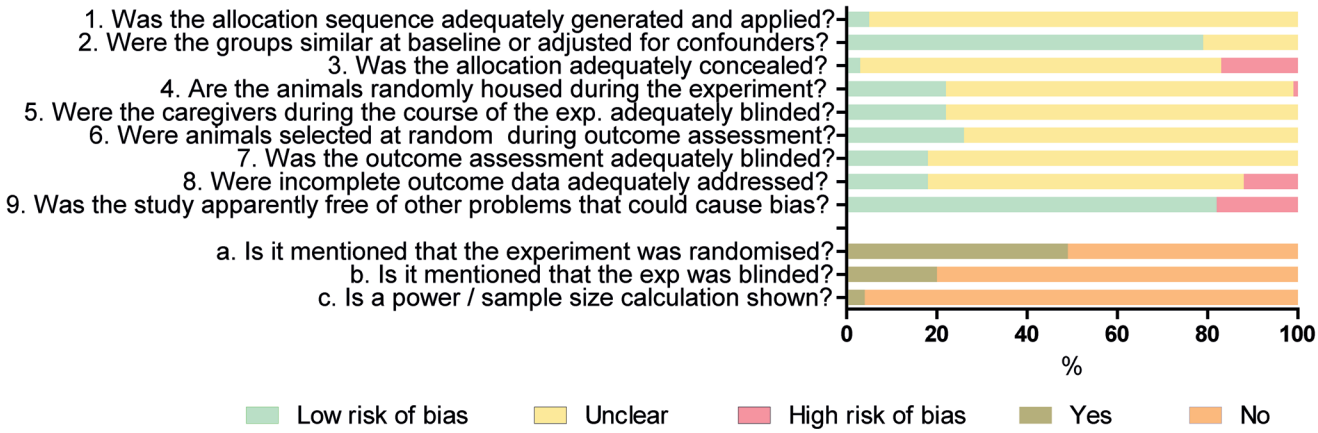

Fig. 3 Risk of bias.

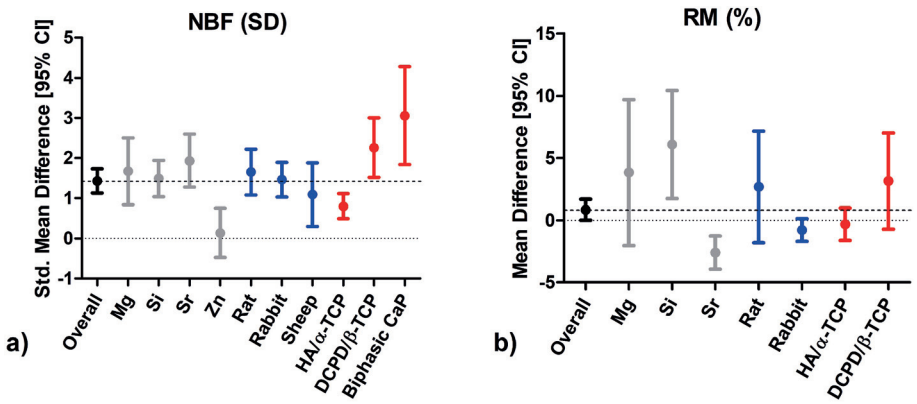

Fig. 4 Meta-analysis of (a) new bone formation (NBF; SD) and (b) remaining material (RM; %). For NBF (SD) the standardized mean difference [95% CI] is plotted and for RM (%) the mean difference [95% CI] is plotted. Black indicator represents the overall effect of bioinorganics supplementation to CaP-based bone substitutes. Subgroup analysis is shown in different colours; bioinorganics in grey, animal species in blue and CaP type in red. Dotted lines represent the 'no effect' value and the dashed lines represent the overall average effect.

Table 3 Subgroup analysis of the included papers for outcome new bone formation (NBF; SD)

Subgroup	Number of comparisons	Number of defects	Effect estimate SMD [95% CI]	Heterogeneity (I^2)
Overall	134	1345	1.43 [1.13, 1.73]	71%
Magnesium, Mg	19	168	1.67 [0.83, 2.50]	66%
Silicon, Si	59	486	1.49 [1.04, 1.95]	57%
Strontium, Sr	27	393	1.93 [1.26, 2.59]	80%
Zinc, Zn	15	162	0.14 [−0.47, 0.76]	66%
Rat	38	417	1.65 [1.08, 2.23]	72%
Rabbit	76	736	1.46 [1.03, 1.89]	73%
Sheep	10	94	1.09 [0.30, 1.88]	50%
HA/α-TCP	85	811	0.87 [0.56, 1.18]	61%
DCPD/β-TCP	36	346	2.26 [1.52, 3.00]	75%
Biphasic CaP	7	96	3.06 [1.84, 4.28]	69%

significantly increased RM, while in 18 comparisons bioinorganic supplementation significantly decreased RM. Regarding type of bioinorganic, Si supplementation significantly increased RM, Mg did not have an effect, and Sr significantly decreased RM ($p < 0.05$ compared to Si and $p > 0.001$ compared to Mg). Bioinorganic supplementation did not have an effect in any animal species nor type of CaP-based bone substitute. Similarly to NBF, subgroup analysis decreased

Table 4 Subgroup analysis of the included papers for outcome remaining material (RM;%)

Subgroup	Number of comparisons	Number of defects	Effect estimate MD [95% CI]	Heterogeneity (I^2)
Overall	57	616	0.84 [0.01, 1.66]	100%
Magnesium, Mg	6	60	3.83 [−2.03, 9.68]	93%
Silicon, Si	27	250	6.08 [1.72, 10.43]	97%
Strontium, Sr	15	180	−2.60 [−3.94, −1.27]	100%
Rat	13	138	2.68 [−1.80, 7.15]	86%
Rabbit	33	348	−0.78 [−1.69, 0.14]	100%
HA/α-TCP	20	202	−0.32 [−1.62, 0.99]	100%
DCPD/β-TCP	33	256	3.15 [−0.71, 7.02]	97%

the heterogeneity, but the decrease does not explain the heterogeneity.

4. Discussion

Supplementation of CaP-based bone substitutes with bioinorganics is a widely researched method to enhance their bone regenerative potential. While in the early 2000s and even before few papers on the topic were published, the past decade has shown a substantial increase in studies on the use of bioinorganics, probably related to an increasing need for effective synthetic bone substitutes. To date, however, no consensus exists regarding the efficacy of bioinorganic supplementation of CaP-based bone substitutes. Here, we systematically reviewed the literature to retrieve papers on the subject and utilized the reported data for a meta-analysis to quantitatively determine the effect of bioinorganic supplementation of CaP-based bone substitutes on bone formation and material degradation. Subgroup analyses were also done to determine the effect of individual bioinorganic supplementation, differences between different types of CaP-based bone substitutes, and animal model effects on these outcome parameters. Our main finding is that bioinorganic supplementation of CaP-based bone substitutes enhances bone formation and affects material degradation in a bioinorganic-compound-dependent manner. Subgroup analyses showed that Sr, Mg and Si significantly enhanced bone formation, while addition of Zn did not have an effect. Bone formation was most enhanced in the more degradable DCPD/β-TCP ceramics and biphasic CaPs, while the effect in the more stable HA/a-TCP was less pronounced. Finally, the effects of bioinorganic supplementation of CaP-based bone substitutes on bone formation and material degradation were similar in different animal models.

4.1. Type of bioinorganic

Bioinorganic supplementation of CaP-based bone substitutes is commonly performed by ionic substitution. Bivalent cations such as Sr^{2+}, Mg^{2+} and Zn^{2+} can substitute Ca^{2+} ions within the crystal lattice of CaP, while CO_3^{2-} as well as SiO_4^{4-} can substitute the phosphate group (*i.e.* PO_4^{3-} or HPO_4^{2-}) and F^- as well as Cl^- can replace the hydroxyl (OH^-) group. Because these bioinorganic ions commonly have different ionic radii than the replaced ones, their supplementation can induce different conformational changes in the crystal lattice structure, which in turn, can lead to changes in crystal lattice stability, microstructure, crystallinity and solubility.[127] This change in the lattice structure can also lead to an increased calcium and/or phosphate release, and more generally, the ion exchange dynamics between the ceramic and a biological system, which can affect processes related to bone formation and remodeling.[128] Thus, the effect of bioinorganics supplementation of CaP-based bone substitutes on bone formation or material degradation is likely related to direct chemical effect of the bioinorganic compound used as well as to changes in the lattice structure of the CaP ceramic resulting from its incorporation. Most studies using bioinorganic supplementation did not show the release of bioinorganics *in vivo*, which leads us to infer that the enhancement in bone formation is also related to the conformational changes induced in the CaPs when supplemented with bioinorganics.[23]

Regarding the effect of bioinorganic supplementation on bone formation, all bioinorganics included in this study improved bone formation, with the exception of Zn. While the exact reason for this positive effect can still not be fully explained, it may be partly related to previous findings that Si plays a role in the initiation of the mineralization process of bone,[17] Mg can increase the alkaline phosphatase (ALP) activity[129] and Sr stimulates osteoblastic activity.[130] Zn, however, has demonstrated to have a concentration-dependent effect on bone formation both *in vitro* and *in vivo*.[127] Ikeuchi *et al.* observed that supplementation with 6.5 μg mL^{-1} of Zn to the culture medium of human bone marrow cells induced mineralization, but higher Zn concentrations hindered it.[131] Similarly, Ito *et al.* reported that CaP ceramics containing up to 1.3 wt% of Zn enhanced the proliferation of MC3T3 osteoblastic cells, while higher concentrations were cytotoxic.[38] In the included animal studies, a wide range of Zn concentrations was used (from 0.04 wt% to 5 wt%). Therefore, it is not surprising that, on average, Zn supplementation had no effect on bone formation due to the inhibitory effect of the higher concentrations. In agreement with this, Kawamura *et al.*[40] observed an increased bone formation when supplementing HA-based scaffolds with 0.316 wt% of Zn, but in scaffolds con-

taining higher Zn concentration, an increased bone resorption was observed. Furthermore, the substitution of Zn towards Ca is restricted to about 15%,[132] while other bioinorganics, such as Sr can fully substitute the Ca.[133] For example, Elgali *et al.* (2016) studied three levels of Ca substitution of HA by Sr, up to 50%.[70] Hence, it can be supposed that the effect of Zn is limited compared to Sr. It is important to mention that comparison between studies regarding the bioinorganic dose is complicated, as different authors express the amount of bioinorganics incorporated with different units (*i.e.* moles or degree of Ca substitution, among others) and, most importantly, many studies do not indicate the specific dose. On the other hand, Cruz *et al.* (2018) performed a qualitative systematic review on the effect of Zn supplementation into CaPs and concluded that Zn supplementation may be an interesting option for enhancing bone repair.[134]

In vivo degradation of CaP materials can be achieved by two different routes: passive degradation by dissolution of the ceramic matrix or active degradation due to cellular interaction. Bioinorganics supplementation may fine-tune degradation by altering either or both of those routes. Our systematic analysis revealed that incorporation of Sr increased material degradation. Because it is known that Sr decreases osteoclast formation and osteoclast resorbing activity,[135,136] a delay in material degradation would be expected. Therefore, we assume that the enhancement of material degradation is related to a change in the lattice structure due to the smaller size of Sr ions compared to Ca.[10] This change in the CaP lattice structure may reduce its stability and enhance the passive degradation of the CaP matrix.

The sub-group analysis revealed that supplementation with Si inhibited material degradation. This finding is in agreement with the hypothesized mechanism of action, as Si has been commonly supplemented to increase CaP stability.[25,137] Further, careful review of the included papers shows that the use of calcium silicate ($CaSiO_3$) was the preferred method for Si supplementation of the CaP ceramic, *i.e.* β-TCP.[89,115,118] While β-TCP or calcium silicate alone degrade relatively fast, the combination of both interfered with their degradation and was found to enhance bone formation. The authors hypothesized that the degradation of β-TCP or calcium silicate occurred too rapidly to provide adequate conditions for bone in-growth. Apparently, the combination of both created the appropriate environment for new bone formation, even when delaying CaP degradation.

Moreover, it was observed that Sr supplementation was used in all the papers dealing with osteoporotic animals, plausibly due to the fact that Sr has shown beneficial effects in the treatment of osteoporosis.[138,139] For example, Sr ranelate, is a commonly used anti-osteoporotic drug.[140,141]

4.2. Type of animal model

An animal model in biomedical research is commonly chosen based on the 3Rs principles as well as practical issues.[142–144] Still, it is known that large animal models can more accurately mimic the clinical situation and, hence, are more adequate to assess the efficacy of bone substitutes.[4] However, a low prevalence of large animal models was observed in the systematic review. Sheep were used in a sufficient number of papers to allow the meta-analysis of new bone formation, but the number of papers was too low for proper meta-analysis of remaining material. Dogs, goats and pigs were also used, but not enough data were available for meta-analysis. Apart from animal species, the defect characteristics are also important for the outcome and clinical relevance of a study. Defect size and defect site (cortical *vs.* trabecular bone) have been proven to affect the bone formation process. Cortical bone has been shown to regenerate slower than trabecular bone when biomaterials are used as graft substitutes.[145,146] Regarding defect size, it is important that the animal studies involve the use of "critical size defects", which cannot heal spontaneously. For example, in case of a rat calvarial model, an 8 mm defect is generally considered critically-sized,[147] although smaller defects up to 5 mm have been also described as critical.[148,149] In the current review, it was observed that some studies used a 5 mm defect,[92,120] while others used a larger defect size of up to 10 mm.[67,95,104]

4.3. Type of CaP-based bone substitute

Based on the classification suggested by LeGeros (2002), CaP-based bone substitutes can be categorized in different groups with different stability/solubility: (1) hydroxyapatite (HA) and α-tricalcium phosphate (α-TCP); (2) biphasic CaPs, and (3) dicalcium phosphate dihydrate (DCPD) and β-tricalcium phosphate (β-TCP).[150] Overall, we observed that bioinorganic supplementation had no effect on material degradation, but enhanced bone formation for all types of CaPs. This effect was more significant for DCPD/β-TCP and biphasic CaPs than for HA/α-TCP. This can be explained due to the faster degradation of DCPD/β-TCP and biphasic CaPs compared to HA/α-TCP.[151–153] Passive CaP degradation is determined by the chemistry and stability of the used calcium phosphate,[154,155] and hence faster degrading bone substitutes (*i.e.* DCPD/β-TCP and biphasic CaPs) release the bioinorganics earlier, which in turn enhances faster bone formation.

4.4. Limitations of the study and clinical relevance

Our search strategy allowed for inclusion of a large number of studies, which gave a detailed overview of the used approaches for supplementing bioinorganics to CaP-based bone substitutes. Moreover, the large number of papers enabled the performance of meta-analysis and sub-group analysis. However, this led to a high statistical heterogeneity, which is related to the considerable experimental variability.[156,157] Therefore, it has to be emphasized that the current findings should not be generalized as subgroup analysis did not reduce the heterogeneity. In order to compensate for experimental variability, a random effect model was used in the meta-analysis. Ideally, all experiments should be performed in a similar manner, but as previously observed in other systematic reviews using pre-clinical studies, this is difficult to achieve.[3,158,159] Another limitation of the systematic review is the low quality of reporting in

pre-clinical papers. Study blinding was seldom reported and randomization was reported in less than half of the included papers. Most papers reported bone formation as a percentage of the defect area but, however, some articles reported it as BMD (mg cm^{-3}) or as a regeneration efficiency ratio, which required to plot the NBF by means of the SMD (in SD, not %). Therefore, it is not known exactly to which extent the bone formation was enhanced (in %). Nevertheless, an estimate of ~8% increase in bone formation would be expected.

Regarding the clinical application of supplementation of bioinorganics, a general screening of literature available was performed in PubMed using a similar search and the 'Clinical Trial' filter. This search showed that only Mg[43–45,160–163] and Si[46–48] have been already supplemented to CaP materials for clinical applications and are being commercialized for oral and orthopedic surgical procedures.[164,165] Although both bioinorganics were shown to enhance bone formation compared to unfilled defects and to the similar extent as xenografts,[161,163] autografts showed a superior performance.[46,166]

5. Conclusion

This systematic review and meta-analysis indicates a significant positive effect on new bone formation by supplementing CaP-based bone substitutes with bioinorganics compared to CaP-based bone substitutes without bioinorganics, especially when using strontium, silicon or magnesium. Moreover, the rapidly degrading DCPD/β-TCP ceramics and biphasic CaPs benefited from bioinorganic supplementation to a higher extent than the slowly degrading HA/α-TCP. Bioinorganic supplementation did not have an overall effect on material degradation, but strontium significantly enhanced and silicon inhibited degradation of synthetic CaP-based bone substitutes. Further research is needed to pinpoint whether these effects on new bone formation and material degradation are directly related to the biological properties of bioinorganics or to the structural changes in CaP-based bone substitutes resulting from supplementation by bioinorganics.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work is financially supported by Life Science & Health (project BONE-IP2), Dutch Ministry of Economic Affairs. P. H. gratefully acknowledges the Gravitation Program "Materials Driven Regeneration", funded by the Netherlands Organization for Scientific Research (024.003.013). The authors want to thank Alice H. J. Tillema for the help with the systematic search strategy development. I. L. T wants to thank Ms Bing Wang, DDS for her help in the translation from Chinese of three of the included papers.

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