10.5005/jp-journals-10024-2306

REVIEW ARTICLE

Biomarkers as Independent Predictors of Bone Regeneration around Biomaterials: A Systematic Review of Literature

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ABSTRACT

Background: Biomarkers are detected during bone formation and resorption associated with the dynamics of bone metabolism and are gaining importance as preferential indicators of bone healing in comparison with conventional methodologies. Current literature suggests that the usage of bone turnover markers for monitoring bone regeneration in association with biomaterials is limited.

Aim: To systematically review literature and evaluate whether bone-biomarkers can independently predict bone regeneration following implantation of various bone biomaterials.

Materials and methods: An electronic search was conducted in PubMed (MEDLINE) database from 1980 to January 2017. The articles for systematic review were selected based on formulated inclusion and exclusion criteria

Results: Upon database searching, 443 articles were retrieved and thoroughly reviewed based on the inclusion and exclusion criteria. In all, 41 studies were finally included for evaluation out of which 4 were clinical studies and the remaining 37 studies utilized animal models. On further evaluation, 12 studies reported the presence of biomarkers in association with cellular response during bone regeneration around biomaterials. Moreover, biomarkers related to enzyme activity and matrix protein derivatives were enhanced during bone-matrix deposition as reported in 14 studies. Inorganic skeletal matrix biomarkers indicative of bone mineralization showed positive expression in eight studies.

Conclusion: Several biomarkers appear to be useful for the assessment of bone regeneration around biomaterials. Although biomarkers are capable of independently predicting bone regeneration, lack of substantial evidence in the literature limits their true clinical utility.

Clinical significance: Noninvasive and inexpensive methods of isolating and characterization of biomarkers from cellular and extracellular skeletal matrix during bone regeneration have proven value in evaluating success of bone biomaterials.

Keywords: Biomarkers, Biomaterials, Bone regeneration, Systematic review.

How to cite this article: Albeshri S, Alblaihess A, Niazy AA, Ramalingam S, Sundar C, Alghamdi HS. Biomarkers as Independent Predictors of Bone Regeneration around Biomaterials: A Systematic Review of Literature. J Contemp Dent Pract 2018;19(5):605-618.

Source of support: Nil

Conflict of interest: None

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BACKGROUND

The prevalence of periodontal disease has increased and it has been recognized as the most common oral disease in recent times. It is characterized by periodontal infection followed by inflammation (periodontitis), leading to destruction of the supporting tooth, periodontal soft tissues, and the dental alveolar bone. The goal of

periodontal therapy is to eliminate infection and inflammation, restore periodontal soft tissues, and stabilize the alveolar bone.[1] A stable and healthy alveolar bone is necessary for the long-term functioning of dental implants (DI) and their corresponding superstructures. Guided tissue regeneration (GTR) and guided bone regeneration (GBR) aim to reconstruct periodontal soft tissues and regenerate damaged alveolar bone respectively, through the application of different biomaterials (i.e., membranes, bone substitutes) over an osseous defect.[2] Guided bone regeneration is capable of regaining the contour of the diseased dental alveolar ridge[3] and is also useful for socket preservation,[4] thereby helping in the replacement of missing teeth with DI.[5] As a result of its predictable benefits, GBR has become an integral part of periodontal therapy and DI rehabilitation procedures.[6]

In clinical practice, bone regeneration and healing are primarily evaluated by radiographic imaging in addition to bone sounding and histopathological evaluation of biopsied bone.[7] There are several reported limitations to the traditional diagnostic methods which make optimal estimation of the success of GBR difficult. Moreover, radiographic determination of bone healing is highly subjective and can prove difficult to diagnose during the early phases of bone regeneration.[8] The advent of bone-biomarkers as an assessment tool with the primary objective of monitoring early bone regeneration is therefore promising. Bone-biomarkers, when evaluated objectively, serve as an indicator of not only the normal bone healing process, but also pathogenic processes and responses to therapeutic intervention. Evidence-based literature acknowledges bone-biomarkers as a noninvasive, convenient, and relatively inexpensive indicator for monitoring bone metabolism and early healing.[9]

Bone-biomarkers indicative of metabolic processes include collagen breakdown products such as hydroxyproline, collagen crosslinks and telopeptides in addition to noncollagenous matrix proteins such as bone sialoprotein (BSP), osteoclast-specific enzyme like tartrate-resistant acid phosphatase (TRAP) and cathepsin K. On the contrary, biomarkers such as alkaline phosphatase (ALP), osteoblast-specific proteins like osteocalcin (OCN) and osteopontin (OPN), and type I collagen (COL-1, byproduct of collagen neosynthesis) are secreted during different stages of bone formation.[10] Similarly, bone-biomarkers are formed as byproducts of bone cell activity during the different phases of bone healing,[11] which begins with early bone cell reactions, followed by bone matrix deposition, and, finally, matrix mineralization and remodeling.[12]

The utility of identifying bone-biomarkers during bone healing not only enhances the accuracy of assessing bone regeneration, but would also allow early detection of successful outcomes.[13] Elevated levels of bone-biomarkers have been clinically detected in the serum and saliva, wherein their quantitative evaluation has proved to be of diagnostic and prognostic significance.[14,15] In spite of their extensive clinical implications, only limited studies have demonstrated the utility of biomarkers as a diagnostic measure of bone regeneration. Even within the limited evidence available in the literature, biomarker evaluation has been considered only as a secondary tool of assessment of bone regeneration, while histopathology, histomorphometry, and radiographic imaging, or a combination of the above has remained the primary choice. Therefore, the objective of this systematic review of literature was to evaluate the role of bone-biomarkers in independently predicting bone regeneration following implantation of various biomaterials.

MATERIALS AND METHODS

Focused Question

The present review of the literature was conducted with the focused question: "Are bone-biomarkers capable of independently predicting bone regeneration following implantation of different bone biomaterials in an osseous defect?"

Literature Search and Selection Criteria

A systematic review of published studies evaluating bone-biomarkers during bone regeneration from 1980 until and including January 2017 was conducted. An electronic search was organized in PubMed (MEDLINE) database using the terms "bone-biomarkers," "bone regeneration," and "bone biomaterials" in combination with the Boolean operators "AND" and "OR."[16] Following this, a manual search was performed additionally by screening the bibliographies of relevant retrieved articles and adding free-text words from titles or abstracts to identify potentially pertinent articles.

All articles retrieved through the literature search were imported into a bibliographic referencing software program (EndNote X7), and duplicate references were identified and removed. In order to eliminate selection bias, two independent reviewers (SA, AA), who were calibrated for intraobserver and interobserver reliability and agreement screened the relevant titles, abstracts, and full texts, and the articles for final review were selected according to preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines,[17] based on the following inclusion and exclusion criteria.

Inclusion Criteria

- Original research articles published in the English language, based on human clinical trials, case–control

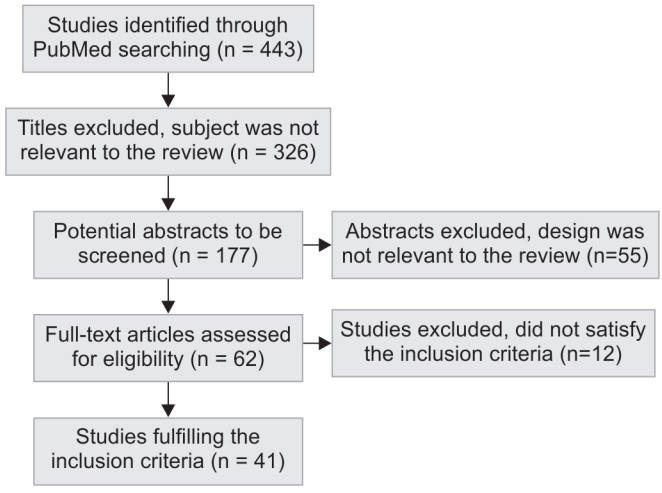
studies, cohort studies, case series, and case reports in addition to *in vivo* animal studies related to the usage of bone-biomarkers for evaluating bone regeneration in osseous defects following placement of biomaterials.

- Articles presenting data pertaining to the model used for research, biomaterials used for bone regeneration, bone-biomarkers evaluated, and the methods used for their assay, along with information relating to follow-up examination protocols.

Exclusion Criteria

Studies with insufficient information, *ex vivo* and *in vitro* researches, case reports, reviews and, technical and personal communications.

Flow Chart 1: Search strategy and articles included in the systematic review



Data Extraction and Study Characteristics

Data extraction from all included studies was independently performed and verified by the two reviewers. When both reviewers agreed on exclusions, the reasons for exclusion were recorded. Any remaining disagreements were resolved by consensus or discussion, if necessary. The data extraction process was guided by a data extraction sheet that specified the relevant study characteristics, including author, year of publication, study design, information related to bone regeneration procedures (type of implanted biomaterials, anatomical site, and healing time), data for the biomarkers assessment (type and assay methods), and reported biomarkers' outcomes that evaluate bone formation in relation to the implanted biomaterials.

RESULTS

A total of 443 studies were identified through electronic searching of the PubMed (MEDLINE) database. Screening the titles and abstracts of the identified studies led to the selection of 117 full-text manuscripts, which were scrutinized and narrowed down to 62 studies, based on their relevance to the focused question of the present review. Following exclusion of duplicates, 41 studies fulfilled the inclusion criteria and were finally considered for systematic review (Flow Chart 1). The selected studies were reviewed by both the authors for the purpose of data extraction, and interpretation. A detailed characterization of the study objectives, study subjects (human/animal model), type of defect investigated along with implanted bone biomaterial, and the biomarkers evaluated under the specified time period for desirable outcomes is elaborated in Table 1.

Description of Experimental Methods

Majority of the reviewed studies used animal models in their research, while only four studies[18-21] were based on human clinical models. While two out of the four human studies, evaluated the role of biomarkers for assessment of bone regeneration,[19,21] one of the studies was based on the assessment of bone formation along with GTR[20] and another study was based on osseointegration and new bone formation around titanium DI.[18] Heterogeneity in terms of the protocols of biomaterial implantation and their respective follow-up periods were widely observed among the reviewed studies. Interestingly, the shortest period of biomaterial implantation, which was evaluated, was 1 day,[22,23] whereas the maximum period of biomaterial implantation was observed to be 16 weeks.[18]

Several bone biomaterials were investigated (e.g., different bone grafts and bone substitutes, membranes, titanium DI with surface modifications, and scaffolds loaded with drugs, osteogenic cells, or biological factors) in different studies. All the included studies reported some degree of bone regeneration based on the assessment of several biomarkers as mentioned in (Table 1). Heterogeneity was observed among the 37 animal studies, in terms of the anatomical sites chosen to recreate an osseous defect for placement of the biomaterials. Several studies were based on a rodent model, out of which one study in mice[24] and another study in rats[25] used maxillary defects. Similarly, mandibular defects were the chosen site in sheep,[26] rabbit,[27] and rat[28-33] models in eight of the included studies. While cranial and calvarial defects in rats were created in four of the reviewed experiments,[34-37] two studies were based on calvarial[22] and spinal[38] defects in mice respectively. Among the other experimental models in rats, femur,[39-41] ulna,[42] tibia,[40,43-45] and intramuscular sites[46] were utilized for the evaluation of bone regeneration.

Nine of the reviewed studies examined the role of biomarkers, based on bone regeneration experiments in rabbit models. The osseous defect sites used in those

Table 1: Characterization of the studies evaluated during systematic review

Author	*Model of study*	*Model of defect*	*Bone biomaterials*	*Type of biomarkers*	*Time of evaluation*	*Method for assessing biomarkers*	*Results*
Sela et al[43]	Rats	Tibia	KG-Cera titanium, Ceramic KGy-213	ALP Phospholipase A2	1, 2, and 3 weeks	IHC	ALP, phospholipase A2 elevated through day 14 and returned to baseline by day 21
Kabashima and Nagata[20]	Human	Periapical teeth defects	PTFE	ALP, OPN IL-4 BMP-2		IHC	ALP and OPN predominantly appeared in the regenerative cells Fewer mononuclear cells (CD4) and IL-4 markers illustrated *in vivo* bone generation
Kamakura et al[36]	Rats	Calvarial	OCP	OCN	4 weeks	IHC	Osteoinductive and osteoconductive nature of OCP with OCN
Itala et al[45]	Rats	Tibia	Bioactive glass micro spheres (Ø 215–350 m)	COL-1 OCN, OPN ONC MMP-9	1, 2, and 8 weeks	RT-PCR	Higher levels of osteoclastic markers like cathepsin K and MMP-9 to COL-1 at 2 and 4 and only MMP-9 at 8 weeks
Shirakura et al[60]	Rats	Maxilla	Sandblasted HA modified Titanium implants	TRAPase	5 and 28 days	IHC	Osteoclast-like cells reactive to TRAPase activity appeared on the implant surface at postoperative 5 days in the HA-group
Aghaloo et al[22]	Mice	Calvaria	Nell-1 protein coated scaffold	CBFA-1 RUNX-2 ALP, OPN BSP, OCN OSX, BMP-7	0, 3, and 6 days	RT-PCR	FGF-2 and TGF-beta1 stimulated Nell-1 expression indicating osteogenesis and reduction of OSX and ALP
Tanaka et al[40]	Rats	Tibia	e-PTFE	cbfa-1 VEGF OCN	6, 8, and 10 days	IHC RT-PCR	Gradual increase in expression of OCN mRNA in control group. Relatively lower expression of VEGF mRNA expression in the experimental group
Yoon et al[52]	Rabbits	Femur	Calcitriol Loaded PLGA scaffold	ALP ONC COL-1	2, 4, and 9 weeks	RT-PCR	An elevated level of ALP, ONC, and COL-1 mRNA was observed at day10
Adeyemo et al[26]	Sheep	Mandible	Onlay bone graft	KI-67 Caspase-3 TUNEL	4, 8, 12, and 16 weeks	IHC	Moderate level of Ki67 at 8 weeks. Caspase-3 on bone surfaces after 16 weeks
Lima et al[21]	Human	Intra bony	e-PTFE	ALP, OPN, OPG, FBGF IL-1,IL-6 MMP-2, MMP-9, OCN	21 days	RT-PCR	Enhanced expression of ALP, OPN, OPG, FBGF, IL-1, IL-6, MMP-2, and MMP-9 in sites where guided tissue regeneration ($p < 0.05$). Decreased levels of OCN No significant changes in IL-4 mRNA levels in test and control groups
Monjo et al[54]	Rabbits	Tibiae	Fluoride modified titanium implant	ALP, OCN LDH, IL-6 RUNX-2 COL-I, IGF-I TNF-α,IL-10	4 and 8 weeks	RT-PCR	Expression of OCN, runx2 and collage type I gene

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Author	*Model of study*	*Model of defect*	*Bone biomaterials*	*Type of biomarkers*	*Time of evaluation*	*Method for assessing biomarkers*	*Results*
Samee et al[46]	Rats	Intramuscular	Human periosteal cells + BMP-2, VEGF	ALP OCN COL-1 CD31+	4 and 8 weeks	RT-PCR IHC	Expressions of biomarkers were higher in BMP-2+VEGF group with enhanced ectopic bone formation at 8 weeks
Alam et al[49]	Rabbits	Nasal bone	Statin/ACS rhBMP-2/ ACS only ACS	BMP-2	1, 2, and 4 weeks	IHC	Statin implanted groups showed more BMP-2 expression than the control group
Jiang et al[32]	Rats	Ramus	Pre mineralized silk scaffolds modified by: bMSCs, AdBMP-2 gene	BMP-2 bMSCs AdBMP-2	8 weeks post insertion	IHC	AdBMP-2 transduced bMSCs implants with higher levels of BMP-2
Kodama et al[24]	Mice	Maxilla	rhFGF2-impregnated gelatin hydrogel	TRAP, OCN RUNX-2 ALP, OPN	4, 6, and 8 weeks	IHC	Bone anabolic activity signaled by FGF/FGFR through simultaneous activation of RUNX-2 and BMP2
Trejo et al[50]	Rabbits	Femur	Silica-based ordered mesoporous SBA15	RUNX-2 OPN, OCN PTH-related protein	4 and 8 weeks	IHC	Osteogenesis was related to increase of Runx2 and OPN, OCN at 4 and 8 weeks
Wohlfahrt et al[55]	Rabbits	Tibia	Titanium implant	RUNX-2 OCN, COL-I TRAP, TNF-α IL-6, ALP 10 LDH	4 weeks	RT-PCR	No significant difference in gene expression between groups
Colombo et al[30]	Rats	Mandible	Titanium implant	OCN OPN TNFα TGF-β	1–12 weeks post-implant insertion	Immuno-cytochemistry	Corresponding to osseointegration changes were observed in the levels of IL-1beta, TNF-alpha, macrophages, and TGF-beta1 in the diabetic bone
Liu et al[47]	Rabbits	Alveolar bone	Autogenous bone	eGFP rhBMP-2	12 weeks	IHC	Detection of transfected genes and rhBMP-2 promoted osteogenic activity
Ratanavaraporn et al[42]	Rats	Ulna	Gelatin hydrogels incorporating combined SDF-1 and BMP-2	Cxcr4 RUNX-2 OCN	12 weeks	RT-PCR	Combined SDF-1 and BMP-2 significantly up-regulated the Cxcr4, Runx2, and OCN expression
Barhanpurkar et al[58]	Mice	subcutaneously	IL-3 with bio graft of HA/TCP+MSC	ALP, OCN OSX, OPN COL-1 RUNX-2 BMP-2		RT-PCR ELISA	Enhanced expression of osteoblast specific genes such as ALP, COL-I, OCN, and OPN and also Runx-2 and osterix transcription factors. JAK/STAT pathway utilized expression of BMP-2, and activation of smad1/5/8
Colombo et al[31]	Rats	Mandible	Roughened titanium implant	PCNA OCN OPN	1, 3, and 9 weeks	IHC	No significant difference in the expression of PCNA, OPN, and OCN among tested implants

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Author	*Model of study*	*Model of defect*	*Bone biomaterials*	*Type of biomarkers*	*Time of evaluation*	*Method for assessing biomarkers*	*Results*
Lu et al[38]	Mice	Spine	Apatite-coated silk fibroin scaffolds	ALP, OCN OPN, BSP BMP	4, 8, and 12 weeks	RT-PCR	Statistically significant ALP activity in AdBMP-2 group. Upregulated mRNA expression of BMP-2, OPN, OCN, and BSP after AdBMP-2 transduction
Osugi et al[29]	Rats	Mandible	Human MSCs/agarose (MSCs), DMEM PBS/ agarose	OCN RUNX-2 COL-1 GADPH	4 and 8 weeks	IHC	Enhanced expression of osteogenic marker genes, such as OCN, Runx2, and of rat MSCs (rMSCs) *in vitro*
Reis-Filho et al[33]	Rats	Mandible	Demineralized human dentin matrix as bone graft	VEGF	3, 7, 14, and 21 days	IHC	DHDM increases the expression of VEGF and accelerates bone healing process
Rios et al[57]	Sheep	Rib	GNAS1 and PHD2 Short-interfering RNA (siRNA)	ALP OCN OPN COL-1	1, 2, 4, 7, 21, 36, and 70 days	ELISA	COL-I and OPN expression was promoted only by siGNAS1
Schulze-Spate et al[19]	Human	Maxillary sinus	β-TCP	TRAP	21–40 weeks	IHC	The total bone was observed to increase, whereas the amount of graft material was found to be diminished
Du et al[44]	Rats	Tibia	Simvastatin modified Titanium implants	ALP BALP BGP	28 and 84 days	IHC	ALP was higher in the OVX group. BALB was higher in osteoporotic rats treated with simvastatin
Monjo et al[53]	Rabbits	Tibiae	Titanium implant	ALP, LDH RUNX-2 COL-1 OCN, IGF-I IL-10,IL-6 CT, TNF-α TRAP	4 and 8 weeks	RT-PCR	OCN and COL-1 expressions were the best predictive markers for osseointegration after 4 and 8 weeks
Moreira et al[27]	Rabbits	Mandible	autologous and allograft	CD31+	3, 7, 14, 28, and 56 days	IHC	CD31+ expression related to osteoblast differentiation and bone matrix synthesis was increased
Pedersen et al[35]	Rats	Calvarial	Poly(L-lactide-co-1,5-dioxepan-2-one) (poly(LLA-co-DXO)) scaffolds	ALP, OCN BSP, IL-1 IL-6 IL-10 CD31+	2, 4, and 8 weeks	RT-PCR IHC	Defects treated with HBO marked with increased number of endothelial CD31+ indicating osteogenesis during the 2nd week
Prati et al[18]	Human	Maxilla and Mandible	IM and NL implants	OPG TGF OCN OPN PTH	7, 15, 30, 60, 90, and 120 days after surgery	LUMINEX/ Magpix system	A preliminary gene release peak of TGF, OPG, OPN, and PTH was elucidated in IM group. Positive modulation of bone mediators was evidenced with immediate loading of the implants

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Author	*Model of study*	*Model of defect*	*Bone biomaterials*	*Type of biomarkers*	*Time of evaluation*	*Method for assessing biomarkers*	*Results*
Taut et al[25]	Rats	Maxilla	Sclerostin-neutralizing monoclonal antibody	OCN PINP TRAP-5b	3, 4, and 6 weeks	OCN Single-plex LINCOplex Kit, ELISA kits	OCN and PINP level were significantly higher in animals treated with Scl-Ab
Zhang et al[56]	Dogs	Femur	Sr-HT Implants with ceramic coating	ITGβ-1 BMP-2 ALP OCN	12 weeks	RT-PCR	*In vitro* mineralization showed Sr-HT group to have the strongest expression of OCN, BMP-2, and ALP
Tera Tde et al[23]	Rats	Mandible	Expanded PTFE, autogenous bone graft	RANK RANKL OPG	0, 7, 21, 45, and 60 days	IHC	The e-PTFE membrane delayed the bone resorption influencing the expression of the biomarkers
Tera Tde et al[28]	Rats	Mandible	Autogenous Bone Graft +e-PTFE	OCN BSP ONC	0, 7, 21, 45, and 60 days	IHC	Bone metabolism was more intense between days 7 and 21 showing more expression in granulation tissue ONC and OCC
Uchida et al[51]	Rabbits	Femur	P-AF	TGF-β TGF-β RIII RUNX-2 COL-1 OCN	1 and 2 weeks	IHC	Significantly higher expression pattern in a set of osteoblast specific genes TGF-beta, TGF-beta type III receptor, Runx2, COL-1, and OCN.
Kunert-Keil et al[34]	Rats	Cranial	BCP and β-TCP	ALP, OCN RUNX-2, COL-1 and 2 IGF1, IGF2 VEGF, MMP-8		IHC RT-PCR	*In vivo* grafting of the BCP evidenced the expression of IGF1, IGF2, VEGF, Col1, Col2, and MMP8 identical to TCP
Pati et al[37]	Rats	Calvaria	ECM-ornamented 3D printed scaffolds	RUNX-2 ALP OCN OPN	7 and 14 days	IHC	Cells cultured in ECM scaffolds showed significantly higher expression of Runx2, ALP, OC, and OPN genes
Tan et al[59]	Rats	Femur	Simvastatin modified Mini-titanium alloy	BMP-2 VEGF VEGF-R2	4 weeks	IHC	Enhanced VEGF, VEGF-R2, BMP-2 expression showing promoted osseointegration, and enhanced implant fixation and evidence of angiogenesis in the target bone
Turri et al[41]	Rat	Femur	Naturally derived resorbable membrane	BMP-2, FGF-2, TGF-beta1 and VEGF	3, 6, and 28 days	PCR, WB, IHC	Enhanced expression of bone recruiting and remodeling cells BMP-2, FGF-2, TGF-beta1, and VEGF in the membrane.

TNF: Tumor necrosis factor; IFN-γ: Interferon-y; PINP: Procollagen IN-terminal peptide; MSCs: Mesenchymal stem cells; rhBMP-2: recombinant human BMP-2; nHA-/PLA: nano-hydroxyapatite/collagen/polyL-lactide; AdBMP-2: adenovirus-mediated bone morphogenic protein-2 gene; Sr-HT: Strontium-substituted hardystonite; P-AF: plasma-irradiated silk fibrin; vWF: von Willebrand factor; BGP: Bone gateway protein; Cxcr4: C-X-C chemokine receptor type 4; Scl-Ab: Sclerostin-neutralizing monoclonal antibody; LDH: Lactate dehydrogenase; ADRCs: Adipose-derived regenerative cells; CTS-K: Cathepsin K; rhFGF-2: recombinant human basic fibroblast growth factor 2; BGLAP: Bone gamma carboxyglutamate protein 1; CT: Calcitonin; Hþ-ATPase: Hydrogen potassium ATPase; ITGβ-1: Integrin beta-1; PTG: Porous titanium granules; WPTG: Oxidized porous titanium granules; DPSCs: Dental pulp stem cells; HBO: Hyperbaric oxygen; IM: Immediately loaded; NL: Nonloaded; WB: Western blot

experiments included the alveolar bone,[47] mandible,[27,48] nasal bone,[49] femur,[50-52] and tibia.[53-55] Furthermore, *in vivo* osseointegration around titanium DI was evaluated in the canine femur,[56] and in the mandible[26] and the ribs[57] in a sheep model. The commonly used biomarker assays were immunohistochemistry (IHC) or reverse transcription polymerase chain reaction (RT-PCR) or a combination of both[34,35,40,46] (Table 1). However, a few studies applied other methodologies such as multiplex bead array assay (LUMINEX)[18,25] and enzyme-linked immunosorbent assay (ELISA)[25,57,58] for analyzing and reporting their results.

Outcomes based on Clinical Studies

Schulze-Spate et al[19] aimed to evaluate the biomarkers of healing process following bone augmentation in the maxillary sinus of patients using beta-tricalcium phosphate (β-TCP). They reported that TRAP staining was significantly associated with a decrease in grafted material and increase in new bone formation. Similarly, Kabashima and Nagata[20] demonstrated interleukin (IL)-4-producing cells to be associated with successful *in vivo* bone regeneration. Evaluating new bone formation and osseointegration around titanium DI, Prati et al[18] reported the presence of transforming growth factors (TGFs), OCN, osteoprotegerin (OPG), OPN, and parathyroid hormone (PTH) during the early phase following implant placement and loading. Interestingly, progressively higher levels of bone-biomarker were recorded during the 7th, 15th, and 30th days, thereby indicating the validity of evaluating biomarkers as a surrogate predictor of the different phases of bone mineralization.[18]

Outcomes based on Translational Studies

Among the criteria evaluated for expression of bone-biomarkers in the reviewed studies based on translational animal models, the least degree of disparity was observed in terms of the types of biomaterials (bone grafts, titanium DI, 3D scaffolds, and other biological derivatives) used for bone regeneration and healing (Table 1). Therefore, further analysis of the data was accomplished based on this aspect of the reviewed studies.

Studies using Bone-grafting Materials

Fourteen out of the 41 studies investigated the presence of bone-biomarkers during the osteoinductive and osteoconductive phases of bone healing when autogenic, allogeneic, or other alloplastic bone substitutes were placed in osseous defect sites (Table 1). Moreira et al[27] illustrated enhanced angiogenesis and expression of cluster of differentiation 31+ (CD31+) associated with osteoblastic differentiation at 8 weeks, when using autogenic and allogeneic onlay bone grafts along with platelet-rich plasma (PRP). Interestingly, early osteoclastic activity demonstrated by OCN expression was followed by Ki-67, caspase-3, and the terminal deoxyribonucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labelling (TUNEL) expression, at 16 weeks, also with onlay bone grafts.[26] Three of the reviewed animal experiments[23,28,40] showed that expanded polytetrafluoroethylene (e-PTFE) membrane, when used along with autogenous bone, delayed resorption of the grafted bone, and histologically exhibited greater expression of OCN, BSP, osteonectin (ONC), OPG, RANK, and receptor activator of nuclear factor (NF)-κB ligand (RANKL), while expressing relatively lower quantities of vascular endothelial growth factor (VEGF) and core binding factor alpha-1 (CBFA-1), possibly attributable to the high remodeling rate.

Interestingly, one of the studies[20] on human dental periapical defects with GTR demonstrated ALP and OCN in fibroblasts-like regenerative cells and IL-4 in adjacent tissues of proven bone regeneration. Similarly, demineralized human dentin matrix (DHDM)[33] represented an efficient grafting material for bone regeneration with increased expression of VEGF and accelerated bone healing. The functional efficacy of the calcium phosphate-based bone substitutes was evaluated using biomarkers in five of the included studies. Bone defect sites grafted with osteoconductive scaffolds like biphasic calcium phosphate (BCP), octa-calcium phosphate (OCP)[36] and β-TCP evidenced an increase in the total bone volume[34] and predominantly expressed ALP, OCN, Runt-related transcription factor 2 (RUNX-2), phosphate-regulating neutral endopeptidase, X-linked (PHEX), collagen (COL-1 and 2), insulin-like growth factor (IGF-1), IGF-2, VEGF, matrix metalloproteinase-8 (MMP-8), and bone morphogenetic protein (BMP). While a dose-dependent increase in osteoblast differentiation and matrix mineralization was observed in IL-3 impregnated mesenchymal stem cells grafted along with hydroxyapatite (HA)/TCP,[58] TRAP-positive osteoclast-like cells[19] and osteoclastic markers cathepsin K and MMP-9[45] were reported on the surfaces of HA, β-TCP, and bioactive glass scaffolds.

Studies evaluating Bone Regeneration around Titanium DIs

The clinical success of titanium DI for esthetic and functional rehabilitation can be substantiated with evidence at the molecular level wherein biomarkers of osseointegration such as OCN and COL-1[30,53,59] have been illustrated during periods ranging from 4 to 8 weeks post-implant placement. In a study based on fluoride-coated titanium DI,[54] the expression of OCN, RUNX-2, and COL-1 correlated with modulatory effects of fluoride upon bone formation/resorption phases at the bone–implant biological

interface. Similarly, simvastatin coating around DI placed in an osteoporotic rat model resulted in enhanced angiogenesis and osseointegration as evidenced by the increased expression of VEGF and bone ALP respectively.[44,59] In contrast, no significant changes in biomarker expression were observed in titanium DI with surface modifications comprising of porous titanium granules,[55] roughening,[31] and TCP/HA coating.[31] Nevertheless, ALP, VEGF-R2, CD31, RUNX-2, OCN, COL-1, TRAP, IL-6, TNF-α, bone alkaline phosphatase (BALP), OPN, and OCN were commonly expressed during osseointegration, thereby indicating their significance in prognostic and clinical performance assessment. On the contrary, bone healing around DI showed an increased matrix vesicle enzyme activity (phospholipase-2) with bone bonding material like KG cera than nonbonding material like KGy-213.[43] However, enhanced or delayed mineralization correlated with the expression of ALP. In a similar study on rat maxilla,[60] increased TRAPase activity was reported for sandblasted DI with ceramic coating. Nonetheless, strontium-substituted hardystonite ceramic coating structure had the strongest expression of OCN, BMP-2, and ALP with increased osseointegration ability in comparison with other ceramic-coated DI.[56]

Studies evaluating Bone Regeneration in Three-dimensional Scaffolds

Encapsulation of bioactive molecules in three-dimensionally (3D) engineered scaffolds not only provides mechanical competence during bone regeneration, but was also coherent with elevated levels of biomarkers such as ALP, ONC, BMP, BSP, RUNX-2, and OPN.[22,32,35,37,38,52] More specifically, calcitriol(1,25[OH]2D3)-loaded porous poly(D,L-lactide-co-glycolide) (PLGA) scaffolds[52] along with mesenchymal stem cells, when used for treating large bone defects, resulted in expression of COL-1 in addition to other biomarkers. Similarly, apatite-coated silk fibroin scaffolds,[38] when tested for ectopic new bone formation, revealed upregulated expression of BMP-2, OPN, OCN, and BSP. Additionally, scaffolds of extracellular matrix (ECM),[37] copolymers of poly(L-lactide-co-1,5-dioxepan-2-one),[35] premineralized silk along with BMP-2 modified bMSCs,[32] simvastatin loaded atelocollagen sponge (ACS),[49] and Nell-1 protein coat[22] led to enhanced bone formation with significant expression of ALP, OCN, BMP, BSP, IL-1, IL-6, IL-10, CD31, RUNX-2, and OPN.

Studies evaluating Bone Formation using Other Proteins and Cell Derivatives

Taut et al[25] assessed the positive therapeutic potential of sclerostin antibody (Scl-Ab) to stimulate alveolar bone regeneration in rats demonstrating concurrent expression of higher levels of OCN and procollagen type I N propeptide (PINP) in sclerostin-neutralizing monoclonal antibody (Scl-Ab) treatment groups. Under similar conditions, plasma-irradiated silk fibrin[51] and osteostatin-loaded silica-based mesoporous SBA15 materials[50] grafted in the rabbit femur resulted in significantly higher expression of TGF-β, TGF-β RIII, RUNX-2, COL-1, and OCN owing to new bone formation. Remarkable bone growth was evidenced in osseous defect sites regenerated with VEGF-transfected mesenchymal stem cells and BMP-2 leading to enhanced expression of ALP, OCN, stromal cell-derived factor 1 (SDF-1), IL-6, COL-1, and CD31+.[46] Similarly, gelatin hydrogel combined with SDF-1, BMP-2,[42] and recombinant Fibroblast growth factor-2 (rhFGF2)[24] led to optimized bone formation with fibroblast growth factor (FGF)/ fibroblast growth factor receptor (FGFR) signaled bone anabolic activity and simultaneous expression of RUNX-2 and BMP-2 biomarkers.

DISCUSSION

Bone is a metabolically active tissue and its regeneration comprises of well-orchestrated series of biological events. This continuous process of bone remodeling involves formation (osteoblasts), resorption (osteoclasts), and maintenance (osteocytes) in a definable and spatial sequence affected by intracellular and extracellular signaling pathways.[61] Currently, there are a plethora of available bone augmentation strategies along with advanced cellular analytical methods for characterization of these bone-forming cells and identification of the transcriptional and translational profiles of genes and proteins encountered.[11] Hence, molecular markers of bone have gained importance in recent times to detect the dynamics of bone during various phases of regeneration.[9] In the present review, several studies demonstrated the efficacy of bone-biomarkers as prognostic indicators for the different stages of bone regeneration, in osseous defect sites, following placement of biomaterials. The commonly expressed biomarker identifiable during each stage of bone regeneration and healing when associated with biomaterials is elaborated in Table 2.

Biomarkers of Bone Turnover

While biochemical indexes are capable of differentiating the biomarkers of bone formation and resorption, a sharp distinction may not be appreciated in clinical scenarios. This is clearly evident in the present systematic review wherein most OC fragments where detected in both matrix deposition and mineralization stages of bone healing[51,53,58] (Table 2). Similarly, BMP-2 and COL-1[23,35,51]

Table 2: Bone-biomarkers related to bone regeneration process

Bone regeneration process	*Related biomarkers*	*References*
Early bone cell reactions	ALP, PCNA, BMP-2, COL-1, PCNA, OPN, RUNX-2, CBFA-1	Rios et al[57] Pedersen et al[35] Barhanpurkar et al[58] Colombo et al[30] Marukawa et al[48] Tanaka et al[40]
Bone matrix deposition	OCN, COL-1, BSP, OPN, CD31+, OCN, BMP-2 and 7, RUNX-2, OSX, CBFA-1, ONC, VEGF	Uchida et al[51] Pedersen et al[35] Barhanpurkar et al[58] Tera Tde et al[23,28] Colombo et al[30] Tanaka et al[40] Moreira et al[27]
Bone mineralization	OCN, OPN, VEGF, CD31+, Ki-67, Caspase-3, TUNEL	Monjo et al[53] Colombo et al[30] Tanaka et al[40] Reis-Filho et al[33] Samee et al[46] Adeyemo et al[26]

were expressed during early cell reactions through matrix deposition phases. Moreover, several of the reported biomarkers of bone turnover could have resulted from the nonskeletal processes and might be present in other tissues influencing their circulating levels.[33]

In clinical practice, implantation of bone biomaterials within osseous defect sites is associated with high degrees of success in relation to bone regeneration and healing.[6] Nevertheless, complications arise in 5 to 10% of patients, making them liable to failed bone regeneration and impaired bone healing.[62] Such complications associated with bone biomaterials could be attributed to several factors including a characteristic of host bone, infected tissue, lack of blood supply, and disturbances to the stability of implanted biomaterials during the healing process.[63] However, the assessment of bone healing via conventional radiographic methods is subjective and is less sensitive in predicting signs of early healing complications.[8] Bone-biomarkers are the products of bone cell activity and are associated with several stages of bone healing. Consequently, bone-biomarkers have been analyzed in many of the reviewed *in vivo* studies for monitoring the process of bone regeneration, and provided an early diagnostic value for possible complications.[64]

Bone healing in response to implanted biomaterials is expected to proceed in three overlapping stages: early bone cell reactions, bone matrix deposition, and bone mineralization.[11] The cellular interaction phase begins immediately after the implantation of biomaterials, which causes initial tissue damage and inflammation for approximately 3 to 4 days. There is evidence of formation of a fibrin-rich clot which acts as a scaffold for different molecular and cellular interactions which mediate angiogenesis. Subsequently, resorption of damaged bone by osteoclasts is observed as a key initiator for the stage of bone formation.[9] The most specific and sensitive biomarker produced by bone resorbing osteoclasts is TRAP.[65] Few other biomarkers of osteoclastic activity include the RANKL and its membrane-bound receptor RANK and OPG, wherein bone resorption is inhibited by OPG when it binds to RANKL.[66] Therefore, the balance between OPG and RANKL primarily regulates osteoclastic activity.[67] In the present systematic review, 12 studies reported that TRAP, OPG, ALP, proliferating cell nuclear antigen (PCNA), BMP-2, BMP-3, BMP-4, COL-1, OPN, RUNX-2, and CBFA-1 were associated with the early stage of cellular response to biomaterials (Table 2). Interestingly, in 4 animal studies [24,25,53,55] TRAP significantly correlated with osteoclast-like activity wherein TRAP5b was detected early in the postoperative immunoassays.

Early Bone Cell Reactions

Bone formation, when assessed at an early stage, not only has significant prognostic value, but could also facilitate confirmation of clinical success as reported by Prati et al.[18] Alkaline phosphatase is an ubiquitous, membrane-bound tetrameric enzyme, commensurate with active remodeling of bone and is validated as a predictive indicator (Table 2) in majority of the reviewed literature.[22,24,34,37,57,58] While Kabashima and Nagata[20] found ALP to be associated with fibroblast-like regeneration, Sela et al[43] demonstrated the peak expression of ALP between 14 and 21 days correlating with primary mineralization in newly formed bone surrounding titanium DI. Furthermore, bone anabolic activity signaled by FGFR through concurrent activation of RUNX-2 and BMP-2 was also reported.[24] Few other biomarkers reportedly expressed in association with osteoblastic reactions include BALP, OCN, PINP, and COL-1.[65]

Bone Matrix Deposition

Bone matrix deposition is evident with the proliferation and differentiation of mesenchymal stem cells into osteoprogenitor cells and subsequently into osteoblasts. Bone formation markers are derived from osteoblasts, mainly during osteoid (bone matrix) synthesis (Table 2). The OCN plays an important role in ECM formation and osteoid mineralization through a negative feedback mechanism. An elevated serum level of OCN has been found during periods of rapid bone turnover and it has therefore been considered a valid biomarker when bone resorption and formation are coupled.[10] Similarly, higher concentrations of OPN were observed in areas of bone formation with simultaneous recruitment and stimulation of macrophages and lymphocytes. Tera Tde et al[28] reported intense bone metabolism associated with increased levels of OCN and OPN in healing osseous defect sites treated with e-PTFE and onlay bone graft.

As one of the most abundant types of collagen in osseous tissue, COL-1 constitutes 90% of the organic matrix. The multitude of osteolytic changes occurring in bone remodeling and collagen degradation facilitates identification of COL-1 as a valuable biomarker of bone turnover. Two of the reviewed studies[29,58] reported enhanced expression of COL-1 with a dose-dependent increase in matrix mineralization. The carboxy-terminal cross-linked telopeptides of COL-1 is not reused during collagen synthesis and are therefore considered as specific markers for bone resorption.[45,53-55,57] During the intermediate stage of bone healing, OCN, COL-1, BSP, OPN, ONC, CD31+, BMP-2 and 7, VEGF, CBFA-1, and osterix (OSX) biomarkers were reportedly identified by 14 studies in the present review.

Bone Mineralization

Approximately 2 weeks following the initial implantation, osteoblasts deposit more woven (matrix) bone within the defect.[68] Nevertheless, bone mineralization/remodeling starts only after adaptation of the morphology of new bone to the original tissue. For bone mineralization, 8 of the reviewed studies reported positive for OCN, OPN, CD31+, Ki-67, caspase-3, and TUNEL biomarkers (Table 2). Therefore, it would be alluring to assume impaired bone healing processes, associated with abnormal expression of these biomarkers.[3] Reis-Filho et al[33] and Kunert-Keil et al[34] reported enhanced VEGF and VEGF-R2 expression correlating with evidence of angiogenesis in the target bone. In contrast, Tanaka et al[40] reported a relatively lower expression of VEGF in new bone formation with GBR after a 10-day follow-up period. Based on the above reviewed studies, while VEGF elicited a chemo attractive effect on primary human osteoblasts and mesenchymal progenitor cells, it was significantly expressed only during the terminal stages of bone formation, preceded by an initial low level of detection. A high level of heterogeneity, possibly attributable to different experimental biomaterials, was observed in the reported expression of biomarkers associated with bone mineralization, which included PCNA, BMP, PINP, RANK, RANKL, TGF, Ki-67, Caspase-3, TUNEL and CD31.

Subject Variability

A variety of translational experimental models were used in the reviewed researches including dogs, sheep, rats, mice and rabbits, with each model considered to be ideal and simulating clinical scenarios. While, canine and sheep models exhibited maximum similarity in terms of outcomes measured, majority of the reviewed literatures were based on rodent models (rats and mice). Similarly, several types of bone defects desirable for mimicking bone regeneration in human bone were reported in the review. Interestingly, most of the studies which employed a craniofacial defect model in the mandible[27-29,32,33,48,60] and the calvarial bone,[22,24,34-36] reported appreciable bone formation and isolation of biomarkers for assessment. The variability of the physical and chemical characteristics of scaffolds, reported in the present review, also had a proven influence on bone regeneration and the related expression of biomarkers. For instance, subcutaneous pockets transplanted with collagen sponge and stem cells[56] reported minimal bone formation, in contrast to defect sites treated with osteoconductive scaffolds. Although the efficacy of bone regeneration can be effectively evaluated by biomarkers of bone turnover, their prognostic importance needs to be substantiated. Similarly, the quantification of bone-biomarkers for studying bone metabolism through IHC assays lacks credible evidence in large-scale population studies, in spite of being comparatively less invasive and cost-effective.[69] Nevertheless, clinical limitations in the use of biomarkers as standardized prognostic tools require continued development in identifying and quantifying more reliable biomarkers of bone healing.[70]

Strengths and Limitations

The major strengths of the present literature review are the systematic search strategy and adherence to PRISMA guidelines. However, a language bias may have influenced the study results, as only English language articles were included in this review. Nevertheless, this systematic review provides valuable insights of bone-biomarkers as prognostic indicators of bone regeneration and healing. The pronounced variability and heterogeneity of bone-biomarkers make it difficult to determine their precise thresholds and hence more observational studies are needed to be carried out to identify the desirable biomarkers. Further validation of biomarkers as independent determinants of bone turn over can be established only with long-term clinical studies.

CONCLUSION

Knowledge of bone biology and their regenerative potential has greatly expanded with advances in molecular biology and research. Major limitations of the scrutinized studies were related to the biological and analytical variability. In this review, several biomarkers were confirmed to be useful for the assessment of bone regeneration and healing around biomaterials. However, there was insufficient evidence to determine whether or not bone-biomarkers can be independently utilized to monitor bone regeneration around biomaterials. Nevertheless, standardization of analytical methods and formalizing

protocols toward specific dominant bone-biomarkers can facilitate future research.

ACKNOWLEDGMENTS

The authors would like to thank the Deanship of Scientific Research and College of Dentistry Research Center (CDRC) at King Saud University, Riyadh, Saudi Arabia, for their help and support.

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