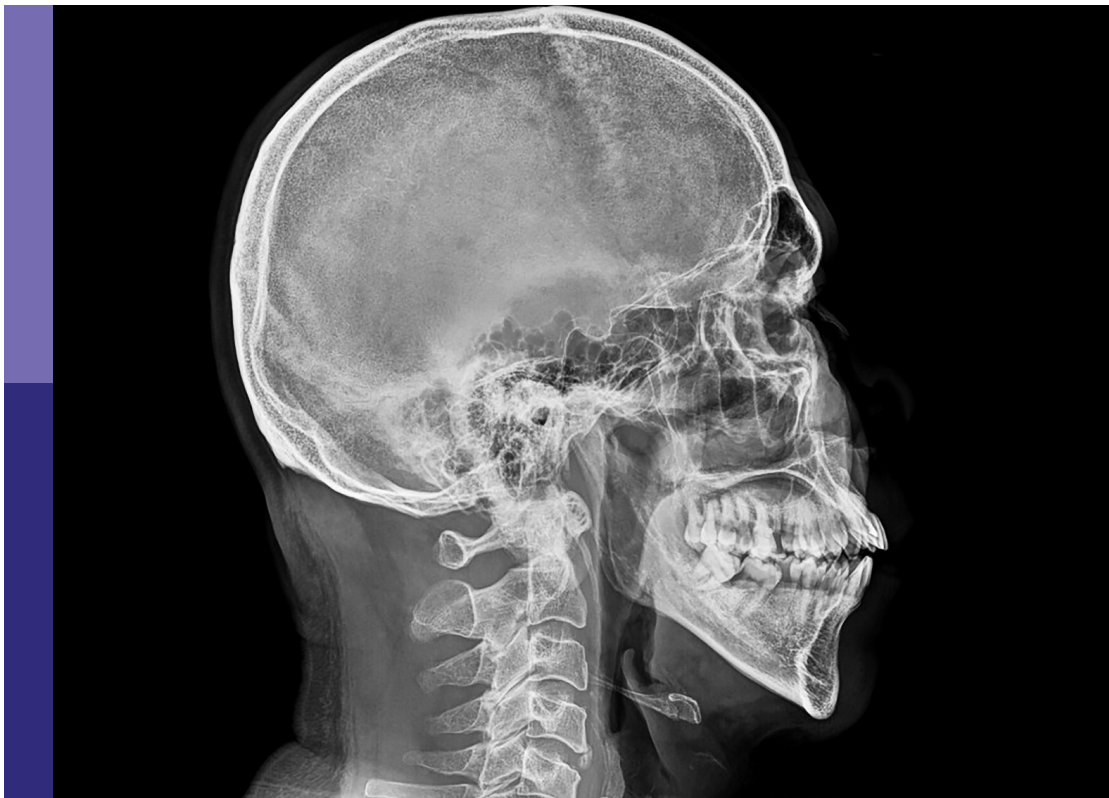
frontiers | Research Topics

# Bone and metabolic activities

**Edited by**
Martha J. Somerman, Kaumudi Joshipura, Francisco Nociti,
Nan E. Hatch, Bjorn Olsen, Thomas E. Van Dyke,
Monzur Murshed and Nancy Lane

**Published in**
Frontiers in Dental Medicine
Frontiers in Oral Health
Frontiers in Endocrinology



ISSN 1664-8714
ISBN 978-2-8325-3208-9
DOI 10.3389/978-2-8325-3208-9

## About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

## Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

## Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

## What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

# Bone and metabolic activities

**Topic editors**

Martha J. Somerman — Field Chief Editor, Frontiers in Dental Medicine, United States

Kaumudi Joshipura — Ahmedabad University, India

Francisco Nociti — ADA Science & Research Institute, American Dental Association, United States

Nan E. Hatch — University of Michigan, United States

Bjorn Olsen — Harvard Medical School, United States

Thomas E. Van Dyke — The Forsyth Institute, United States

Monzur Murshed — McGill University, Canada

Nancy Lane — UC Davis Health, United States

**Citation**

Somerman, M. J., Joshipura, K., Nociti, F., Hatch, N. E., Olsen, B., Van Dyke, T. E., Murshed, M., Lane, N., eds. (2023). *Bone and metabolic activities*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-3208-9

# Table of contents

frontiers | Frontiers in Dental Medicine

TYPE Editorial
PUBLISHED 26 July 2023
DOI 10.3389/fdmed.2023.1244033



OPEN ACCESS

EDITED AND REVIEWED BY
Man-Kyo Chung,
University of Maryland, United States

*CORRESPONDENCE
M. J. Somerman
msomerman13@gmail.com

RECEIVED 21 June 2023
ACCEPTED 10 July 2023
PUBLISHED 26 July 2023

CITATION
Van Dyke TE, Hatch NE, Joshipura K, Lane NE, Murshed M, Nociti F, Olsen B and Somerman MJ (2023) Editorial: Bone and metabolic activities.
Front. Dent. Med 4:1244033.
doi: 10.3389/fdmed.2023.1244033



# Editorial: Bone and metabolic activities

T. E. Van Dyke[1,2], N. E. Hatch[3], K. Joshipura[4], N. E. Lane[5], M. Murshed[6], F. Nociti[7], B. Olsen[2] and M. J. Somerman[8]*

[1]Department of Applied Oral Sciences, Forsyth Institute, Cambridge, MA, United States, [2]Faculty of Medicine, Harvard University, Boston, MA, United States, [3]Department of Orthodontics and Pediatric Dentistry, School of Dentistry, University of Michigan, Ann Arbor, MI, United States, [4]School of Public Health, Ahmedabad University, Ahmedabad, India, [5]Department of Medicine, University of California at Davis School of Medicine, Sacramento, CA, United States, [6]Division of Endocrinology and Metabolism, McGill, Montreal, QC, Canada, [7]American Dental Association Science and Research Institute, ADA, Gaithersburg, MD, United States, [8]National Institute of Dental Craniofacial Research, National Institutes of Health, Bethesda, MD, United States

KEYWORDS

bone, systems biology, endocrine, osteology, transgenic mice, metabolism

Editorial on the Research Topic
Bone and metabolic activities

## 1. Introduction

Substantial progress has been made in defining genes and proteins involved in the development, maintenance and regeneration of teeth and bones. This knowledge has improved strategies for both the diagnosis and treatment of mineralized tissue diseases. Existing data provide credence for these genes/proteins to have roles beyond those attributed to mineralized tissues, including acting as endocrine factors, and altering metabolic activity at distant sites (1). However, there remains substantial uncertainty as to what extent bone itself functioning as an endocrine organ, and/or factors secreted by bone, modulate metabolic activity. This Research Topic was developed to advance our understanding of the effect of hard tissues on metabolic activity, to provide information of value in considering clinical strategies to prevent and treat disorders of mineralized tissue and metabolic activity. This Research Topic is comprised of original research articles, reviews, mini-reviews and perspectives that support the influence of mineralized tissues on metabolism.

## 2. Original articles (4)

There is strong evidence supporting bidirectional effects of chronic periodontal disease and diabetes mellitus on overall health status (2). Further, diabetes is associated with higher risk of long bone and jaw fractures. Two original articles focus on diabetes. In the manuscript by Heikkilä et al., in a ten year follow up study using an impressive cohort of 68,273 compromised patients, they provide additional data in support of an association between chronic oral diseases and diabetes. The inability of individuals with diabetes to regulate insulin levels and related glucose abnormalities is known to compromise the health of numerous tissues of the body including teeth and bones, plus downstream effects on whole body metabolic activity.

In the article by Alharbi and Graves, they induced diabetes in mice with a targeted FOX01 deletion in chondrocytes vs. controls. The results emphasize the important role of FOX01 in modulating a variety of factors associated with a diabetic profile. In another study by Zebrowitz et al., using a mouse model of periodontal disease, periodontally diseased teeth were treated with a timed-release formulation of a small molecule inhibitor of prolylhydroxylases (PHDi; 1,4-DPCA), previously shown to induce epimorphic regeneration of soft tissue in non-regenerating mice. PHDi induces high expression of HIF-1α, a target gene for 1,4-DPCA, and is able to shift the cellular metabolic state from oxidative phosphorylation to aerobic glycolysis, an energetic state used by mesenchymal stem cells and embryonic tissue. The authors showed evidence of metabolic reprograming by increased expression of HIF-1α, as well as metabolic genes *Glut-1, Gapdh, Pdk1, Pgk1* and *Ldh-a* in periodontal tissues.

The data provided in the manuscript by Nagasaki et al., using a mouse model where the arginine-glycine-aspartic acid (RGD) region of bone sialoprotein is replaced by the nonfunctional sequence of lysine, alanine, glutamic acid (KAE knockin), reported that KAEKI mice vs. control mice developed mild obesity, an increase in body weight, adipocyte hypertrophy in white epididymal fat and interscapular brown fat, dyslipidemia and hyperleptinemia but no significant changes in glucose metabolism suggesting that the RGD region of BSP affects energy metabolism by regulating food intake.

### 2.1. Reviews (1)

In a comprehensive review by Franceschi et al. on the role of discoidin domain receptors in controlling bone development, regeneration and metabolism, they provide evidence that in addition to the know interactions of specific β1 integrins and collagen receptors in bone, a second, more primordial collagen receptor family, the discoidin domain receptors also play a critical role in mineralized tissue development as well as related functions in abnormal bone formation, regeneration and metabolism.

### 2.2. Mini Reviews (2)

Two mini reviews center on periodontal tissues, including one by Tazawa et al. on the role of IL-1 signaling in development of apical periodontitis (AP) and the other by Abdalla and Van Dyke on the effect of the soluble expoxide hydrolase (eHS) cascade on periodontal tissues. Tazawa et al. review evidence in support of previous data linking increased alkaline phosphatase enzyme (AP) with obesity and specifically the role of IL-1RA in regulating IL-1 signaling and modulating apical lesion progression in obesity. Abdalla and Van Dyke discuss the mechanism by which eHS inhibitors enhance the production of pro-resolving mediators to affect periodontitis, and further that such knowledge may inspire novel approaches to prevent and treat periodontal diseases.

### 2.3. Perspectives (2)

Nagaski et al. discuss the mounting evidence of the role mineralized tissues and associated factors in controlling whole-body metabolism, including metabolic disorders such as diabetes and obesity, while Fraser and Ganesan provide new insights as to the significance of interactions between oral and gut microbiome, and alveolar bone and associated metabolites in health and disease. They highlight that the advances in metabolomics, transcriptomic and metagenomic technologies should assist in identifying novel metabolites affecting the health of mineralized tissues.

Together, this diverse set of articles invites the reader to consider their own research areas and to rethink, if not already reimagining, the potentially significant role of bones and teeth in influencing metabolic activity in health and in diseased states. We welcome additional articles in this field for publication in FMED or FOH.

## Author contributions

All the co-editors agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

## Acknowledgments

The editors wish to thank the contributions to this Research Topic and the staff at Frontiers in Dental Medicine and Frontiers in Oral Health for their support.

## Conflict of interest

The authors MJS, KJ, FN, NEH, BO, TEVD, MM, NL declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

## References

1. Collins MT, Marcucci G, Anders HJ, Beltrami G, Cauley JA, Ebeling PR, et al. Skeletal and extraskeletal disorders of biomineralization. *Nat Rev Endocrinol*. (2022) 18(8):473–89. doi: 10.1038/s41574-022-00682-7

2. Borgnakke WS, Poudel P. Diabetes and oral health: summary of current scientific evidence for why transdisciplinary collaboration is needed. *Front Dent Med*. (2021) 2:709831. doi: 10.3389/fdmed.2021.709831

frontiers | Frontiers in Dental Medicine

TYPE Perspective
PUBLISHED 27 July 2022
DOI 10.3389/fdmed.2022.974862



OPEN ACCESS

EDITED BY
Thanaphum Osathanon,
Chulalongkorn University, Thailand

REVIEWED BY
Yorimasa Ogata,
Nihon University, Japan

*CORRESPONDENCE
Martha J. Somerman
martha.somerman@nih.gov

SPECIALTY SECTION
This article was submitted to Systems Integration, a section of the journal Frontiers in Dental Medicine

RECEIVED 21 June 2022
ACCEPTED 04 July 2022
PUBLISHED 27 July 2022

CITATION
Nagasaki K, Gavrilova O, Hajishengallis G and Somerman MJ (2022) Does the RGD region of certain proteins affect metabolic activity?. *Front. Dent. Med.* 3:974862. doi: 10.3389/fdmed.2022.974862



# Does the RGD region of certain proteins affect metabolic activity?

Karin Nagasaki[1,2], Oksana Gavrilova[3], George Hajishengallis[4] and Martha J. Somerman[1]*

[1]Laboratory of Oral Connective Tissue Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), National Institutes of Health (NIH), Bethesda, MD, United States, [2]Department of Periodontology and Endodontology, Tohoku University Graduate School of Dentistry, Sendai, Japan, [3]Mouse Metabolism Core, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health, Bethesda, MD, United States, [4]Department of Basic and Translational Sciences, Penn Dental Medicine, University of Pennsylvania, Philadelphia, PA, United States

A better understanding of the role of mineralized tissues and their associated factors in governing whole-body metabolism should be of value toward informing clinical strategies to treat mineralized tissue and metabolic disorders, such as diabetes and obesity. This perspective provides evidence suggesting a role for the arginine-glycine-aspartic acid (RGD) region, a sequence identified in several proteins secreted by bone cells, as well as other cells, in modulating systemic metabolic activity. We focus on (a) two of the SIBLING (**s**mall **i**ntegrin-**b**inding **li**gand, **N**-linked **g**lycoprotein) family genes/proteins, bone sialoprotein (BSP) and osteopontin (OPN), (b) insulin-like growth factor-binding protein-1 & 2 (IGFBP-1, IGFBP-2) and (c) developmental endothelial locus 1 (DEL1) and milk fat globule–EGF factor-8 (MFG-E8). In addition, for our readers to appreciate the mounting evidence that a multitude of bone secreted factors affect the activity of other tissues, we provide a brief overview of other proteins, to include fibroblast growth factor 23 (FGF23), phosphatase orphan 1 (PHOSPHO1), osteocalcin (OCN/BGLAP), tissue non-specific alkaline phosphatase (TNAP) and acidic serine aspartic-rich MEPE-associated motif (ASARM), along with known/suggested functions of these factors in influencing energy metabolism.

KEYWORDS

**bone, mineralized tissues, arginine-glycine-aspartic acid (RGD), metabolic activity, endocrinology, obesity**

## Introduction

Existing data provide credible evidence that proteins produced by mineralized tissues affect the activity of tissues at distant sites (1–4). However, the specific role of these proteins at distant sites has been elusive, with supportive evidence that factors secreted by skeletal tissues may modulate metabolic activity. A better understanding of the role of mineralized tissues and their associated factors in governing whole-body metabolism should be of value toward informing clinical strategies to treat mineralized tissue and metabolic disorders, such as diabetes and obesity.

This perspective provides evidence suggesting a role for the arginine-glycine-aspartic acid (RGD) region, a sequence identified in several proteins secreted by bone cells, as well as other cells, in modulating systemic metabolic activity. We focus on a) two of the SIBLING (**s**mall **i**ntegrin-**b**inding **li**gand, **N**-linked **g**lycoprotein) family genes/proteins, bone sialoprotein (BSP) and osteopontin (OPN), (b) insulin-like growth factor-binding protein-1 & 2 (IGFBP-1, IGFBP-2) and (c) developmental endothelial locus 1 (DEL1) and milk fat globule–EGF factor-8 (MFG-E8) (Table 1 and below). In addition, for our readers to appreciate the mounting evidence that a multitude of bone secreted factors, not just those with RGD regions, affect the activity of other tissues, we provide a table (Table 2) to include the proteins, fibroblast growth factor 23 (FGF23), phosphatase orphan 1 (PHOSPHO1), osteocalcin (OCN/BGLAP), tissue non-specific alkaline phosphatase (TNAP) and acidic serine aspartic-rich MEPE-associated motif (ASARM), along with known/suggested functions of these factors, with an emphasis on their role in monitoring metabolic activity. This latter table provides a brief review with references provided for more detailed information.

# Known factors and candidates for regulating metabolic activity

## Current understanding of factors/environments influencing systemic metabolic activity

Nutrient metabolism is essential for the survival, growth, and development of all living systems. The whole-body metabolic homeostasis of higher organisms relies on precise sensing of the energy state of the body and coordinated response of multiple organs to nutritional demands and environmental changes. The central nervous system plays an important role in regulating all aspects of metabolism, including energy intake, absorption, utilization, and storage (50, 51). One-way key metabolic tissues, such as the liver, muscle, adipose tissue, pancreas, and gut, communicate with the brain and each other is *via* secreted factors, including protein hormones, cytokines, small molecules, metabolites, and extracellular vesicles (52–57). Dysregulation of these inter-organ communications contributes to the pathogenesis of metabolic diseases, including obesity, type 2 diabetes, dyslipidemia, fatty liver disease, and cardiovascular diseases.

Increasing evidence suggests that adipose tissues play a central role in systemic metabolism, providing storage and release of energy from white fat, expending energy to generate heat in brown and beige fat, and secreting a diverse group of bioactive mediators, collectively called "adipokines" (53, 58, 59). Adipose tissue dysfunction in genetic and high-fat diets induced obesity or in lipodystrophy causes toxic lipid accumulation in the liver, skeletal muscle, and other tissues, leading to systemic insulin resistance (60, 61). Adipose tissue inflammation and misbalance between secretion of pro- and anti-inflammatory adipokines also contribute to the pathogenesis of metabolic dysfunction in obesity (62, 63).

Many studies have shown that alterations of glucose and lipid metabolism influence bone homeostasis (64). Skeletal tissue growth and remodeling are energy consuming processes tightly coupled with the regulation of systemic energy metabolism and reproduction (65). Numerous hormones, such as estrogen, testosterone, parathyroid hormone, insulin, adipokines (e.g., leptin, resistin, adiponectin, TNFα), vitamin D, as well as neuropeptides modulate bone metabolic activity (4, 54, 64, 66–70). Recent studies have emphasized the role of the bone marrow adipose tissue, located in close proximity to skeletal lineage cells, in bone metabolism (71–74). Expansion of this depot, observed in aging, obesity, diabetes, anorexia nervosa, is often inversely associated with bone mineral density. As bone marrow adipocytes and osteoblasts share a common precursor, mesenchymal stem cells, imbalance between adipogenesis and osteogenesis may contribute to bone loss under pathological conditions. This broad overview underscores the complex interactions between tissues required to modulate metabolic activity including mineralized tissues, the focus of this perspective.

## Known and proposed functions of the RGD region in proteins

Before moving forward with the major focus of this perspective, the potential role of the tripeptide motif, RGD, in regulating metabolic activity, a brief review of the activities known and proposed for RGD-integrin-binding mediated cell functions is provided for contextual purposes. The readers are referred to a few of many excellent reviews, and references therein: (75–77).

The interest in defining the functions of RGD peptides and associated integrins was spurred on by early studies suggesting that such molecules may serve as therapeutic targets for numerous diseases. These studies demonstrated that the RGD region of proteins *via* interactions with their selective cell surface integrins promotes cell migration, adhesion, and signal transduction, with changes in cell proliferation and differentiation over a life span. Further investigations have proposed roles for RGD-integrin binding to include but not limited to modulating cancer cells e.g., progression, metastasis, angiogenesis, to controlling diseases such as sepsis, fibrosis, neurological disorders, cardiovascular diseases, and viral infections, to monitoring disease progression (diagnostic/imaging tools) and controlling wound healing. At a mechanistic level, studies have shown that in addition

TABLE 1 RGD proteins and metabolic activity.

| Protein | Tisssue/cell expression | Known function | Proposed metabolic function | Integrin receptor | References |
|---|---|---|---|---|---|
| BSP | Bones and teeth (osteoblasts, cementoblasts, osteoclasts) Tumors (breast, prostate, lung cancer cells) | Promotes mineralization. Promotes cell migration and adhesion. Promotes cancer progression and bone-metastasis. | Regulates food intake and energy metabolism. | αvβ3 | Ballahcène et al. (5), Ballahcène et al. (6), Foster et al. (7), Wu et al. (8), Chen et al. (9) |
| OPN | Bones and teeth (osteoblasts, cementoblasts, osteoclasts) Kidneys (tubular epithelial cells) Adipose tissue (activated macrophages) Dendritic, lymphoid, mononuclear cells Tumors (breast, prostate, lung, colorectal cancer) Endothelial cells, Smooth muscle cells, Epithelial cells | Inhibits mineralization. Promotes osteoclastogenesis. Promotes cell migration and adhesion. Promotes cancer progression and metastasis. | Promotes adipose tissue macrophage infiltration. Affects insulin responsiveness. | αvβ3, αvβ5, αvβ1 α4β1, α8β1, α9β1 | Foster et al. (10), Nomiyama et al. (11), Kiefer et al. (12), Dai et al. (13), Zhao et al. (14) |
| IGFBP-1 | Liver, Kidney, Decidua Subcutaneous adipose tissue | Inhibits IGF action. Regulates insulin sensitivity. | Modulates insulin-sensitizing actions. | α5β1, α5β3 | Hoeflich et al. (15), Haywood et al. (16) |
| IGFBP-2 | Embryonic and fetal tissues Multiple tumors (glioma, pancreatic, ovarian, breast, prostate, lung, liver cancer) Adipose tissue | Regulates IGF function. Regulates embriogenesis. Regulates tumorigenesis. | Regulates glucose clearance. Regulates GLUT4 translocation in the muscle. Regulates blood glucose metabolism. | αvβ3, α5β1 | Hoeflich et al. (15), Li et al. (17), Reyer et al. (18), Zhang et al. (19) |
| MFG-E8 | Ubiquitous pattern of expression in different cells and tissues; first identified in mammary gland, also expressed in spleen, gut, lung and adipose tissue | Promotes efferocytosis. Promotes mucosal tissue healing. Inhibits osteoclastogenesis. Mitigates endoplasmic reticulum stress. | Promotes fatty acid uptake. | αvβ3, αvβ5 | Aziz et al. (20), Bu et al. (21), Abe et al. (22), Khalifeh-Soltani et al. (23), Ren et al. (24) |
| DEL-1 | Restricted pattern of expression, e.g., endothelial cells, MSCs, certain macrophage subsets, brain, lung, gingiva, adipose tissue; no expression in liver and spleen | Inhibits neutrophil recruitment. Inhibits osteoclastogenesis. Promotes osteogenesis. Promotes efferocytosis. Regulates Treg cell stability and function. | Might regulate metabolism in a manner similar to MFG-E8 based on similar structure and engagement of the same integrins. | αvβ3, αvβ5, αLβ2, αMβ2 | Hajishengallis & Chavakis (25), Kourtzelis et al. (26), Li et al. (27), Yuh et al. (28), Shin et al. (29) |



10.3389/fdmed.2022.974862

TABLE 2 Non-RGD proteins and metabolic activity.

| Protein | Tisssue/cell expression | Known function | Proposed metabolic function | Receptor/Substrate | References |
|---|---|---|---|---|---|
| TNAP | Expressed in many tissues; Bone, Teeth, Growth plate cartilage, Liver, Bile, Kidney, Intestinal lumen, Brain, Adipose tissue | Promotes mineralization by generating Pi from PPi and other factors.<br>Mutations in the gene (*Alpl*) encoding TNAP lead to hyphosphatasia (HPP), of variable severity from lethal to odontohyphosphatasia (effects limited to teeth/periodontium). | Controll metabolic syndrome (MetS) and associated with enhanced cardiovascular mortality by exacerbating vascular calcification.<br>Modulate bone marrow mesenschymal progenitor cell differentiation toward adipocytes. Promotes purinergic signaling.<br>Modulate inflammatory pathways.<br>Adipocyte-TNAP plays a role in thermogenesis. | Pyrophosphate Pyridoxal 5-phosphate (PLP)<br>Phosphoethanolamine (PEA)<br>LRP6/GSK3beta complex<br>Phosphocreatine<br>OPN<br>Lipopolysaccharides (LPS)<br>Polynucleotides<br>Toll-like receptor ligands and others | Goettsch et al. (3), Millán et al. (30), Briolay et al. (31), Sun et al. (32), Bessueille et al. (33), Krishnamurthy et al. (34), Graser et al. (35) |
| FGF23 | Osteocytes, Osteoblasts | FGF23, a known hormone, acts (as a complex with FGFR/α-Klotho) in the renal proximal tubules to regulate phosphate reabsorption and 1,25(OH)2D3 metabolism and in the distal tubules to modulate sodium and calcium reabsorption. | Propsed functions include suppression of osteiblast differentiation and matrix minerlaization. As stated under know functions, as a hormone it effects overall metabolic activity. | KLOTHO (a co-receptor)<br>FGF receptor 1 (FGFR1) | Bacchetta et al. (36), Bhattacharyya et al. (37), Minisola et al. (38) |
| OCN | Osteoblasts, Cementoblasts | A marker of osteoblast differentiation.<br>Monitors bone formation. | Regulates energy metabolism (glucose regulation and insulin signaling) *via* effects on adipocytes, hepatocytes and pancreatic beta cells. | LRP5<br>Leptin<br>DMP1-ASARM<br>PHEX/IR (insulin receptor) | Wei et al. (1), Confavreux et al. (2), Fulzele et al. (4), Wei et al. (39), Ferron et al. (40), Ferron et al. (41), Ducy et al. (42) |

*(Continued)*





10.3389/fdmed.2022.974862

TABLE 2 Continued

| Protein | Tisssue/cell expression | Known function | Proposed metabolic function | Receptor/Substrate | References |
|---|---|---|---|---|---|
| PHOSPHO1 | Chondrocytes, Osteoblasts, Odontoblasts, Cementoblasts | Modulates skeletal, dentin and cellular cementum mineralization. Initiates deposition of hydroxyapatite inside cell-derived membrane-limited matrix vesicles (MVs) by generating Pi from hydrolysis of MV membrane products. | Regulates insulin responsiveness and obesity (acting in an endocrine fashion). | Phosphoethanolamine Phosphocholine | Suchacki et al. (43), Zweifler et al. (44), Houston et al. (45), McKee et al. (46) |
| ASARM | Osteoblasts, Odontoblasts | Inhibits mineralization. The MEPE (matrix extracellular phosphoglycoprotein)-ASARM is a known substrates for PHEX, with data suggesting that the ASARM motif regulates expression of FGF23, a key factor in monitoring levels of phosphate in the blood. | Defective regulation of ASARM affects energy metabolism, resulting in changes in fat mass, weight, insulin sensitivity, levels of leptin, serotonin and aldosterone, sympathetic tone and vascularization. | FGF23 MEPE DMP1 OCN | Rowe et al. (47), Rowe et al. (48), Salmon et al. (49) |



10.3389/fdmed.2022.974862

to RGD-integrin binding mediating adhesion to extracellular matrix molecules, this interaction results in bidirectional signaling across membranes thus functioning as controllers of cellular processing (78, 79). Some interactions result in multiprotein clustering, forming focal adhesions, the sites of integrin-facilitated cell signaling. This elicits a cascade of phosphorylating events of downstream molecules, most notably focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK), phosphoinositide kinase (PI3K)/Akt, and extracellular signal regulated kinase (ERK) (76, 77). This brief synopsis, demonstrating the varied mechanistic aspects of RGD-integrin mediated cell behavior, sets the stage for the discussion below, to consider how specific proteins and linked integrins, may influence metabolic activity, both locally and at distant sites. Below we consider six RGD containing proteins and their known and proposed functions in modulating metabolic activity, summarized in Table 1 (8, 9, 14, 19).

## RGD containing proteins selective to mineralized tissues proposed as modulators of local and systemic metabolic activity

### OPN and BSP

In the late 1900s the role of the RGD region of proteins, specifically OPN and BSP, found in high concentrations in mineralized tissues and their associated receptors, primarily integrins, was receiving notable attention. Since these early years there have been considerable advances in defining the function of the RGD region in proteins of mineralized tissues, as well as other tissues.

As members of the SIBLING family, OPN and BSP contain several highly conserved functional motifs, including a C-terminal RGD-integrin binding domain known to promote cell adhesion, migration and signaling (5, 6, 80, 81). OPN and BSP, first identified in bone, were proposed to modulate mineralization, where OPN is considered to act as an inhibitor of nucleation and BSP as the crystal nucleator (10, 82–89). It is now recognized that the expression of these genes/proteins is not limited to mineralized tissues, as discussed below.

OPN: In addition to OPN's role *via* the RGD region in modulating cell behavior, OPN's inhibitory effect on mineralization has been ascribed to its negatively charged phosphorylated serine residues, to its modulation of osteoclasts, and to its ability to regulate pyrophosphate metabolism (90, 91). Soon after the discovery of OPN it was found to be expressed ubiquitously and to exhibit a variety of functions dependent on the specific cells/tissues/organs expressing OPN. Relevant to this perspective, OPN has been shown to have a role within bone as a regulator of bone mass (92), as well as a role in modulating systemic metabolic activity as described below.

OPN, also considered as a pro-inflammatory cytokine, has been shown to modulate immune cell responses, with an important role in advancing inflammation within many tissues including adipose, cardiovascular and renal tissues, and associated with obesity, insulin resistance and type 2 diabetes (11, 12, 93–96). In this regard Dai and co-workers provided data revealing the intricacies in defining the role of OPN as a regulator of bone metabolic activity vs. systemic metabolic activity (13). Data from their mouse studies demonstrate that OPN is secreted by adipose tissue (epididymal white adipose tissues) macrophages and selectively circulates to bone marrow, activating osteoclasts to degrade bone and modulating bone marrow-derived macrophages. In the latter case, the macrophages engulf lipid deposits released from adipocytes in the bone marrow and through a complex array of elegantly designed studies the authors showed that OPN-neutralizing antibodies ameliorated high fat diet assisted bone loss in these mice. Mouse studies focused on determining OPN's role in metabolic dysregulation in the liver found that OPN regulated cross talk between cholesterol (CHOL) and phosphatidylcholine (PC) metabolism *via* enhancing expression of cholesterol 7 alpha-hydroxylase (CYP7A1). This finding suggested that OPN, by disrupting PC and CHOL metabolism, may contribute to non–alcoholic fatty liver progression in non-obese patients (97). Further studies by other groups confirmed a role for OPN in modulating age-related non–alcoholic fatty liver disease (98). These studies provide evidence that OPN, regardless of where expressed, affects metabolic activity *via* pro-inflammatory events, and in many cases through integrin signaling pathways; however, a specific role of the RGD region in mediating these events was not discussed. In a study by Chen et al., focused on determining the factors involved in differentiation of mesenchymal precursor cells along an adipogenesis vs. osteogenesis pathway, the authors provided evidence that OPN-integrin links (*via* $\alpha v\beta 1$) may be involved in this process (99). They further showed that the ratio of total fat weight to body weight was significantly higher in OPN-deficient mice at 5 weeks and 12 months vs. wild-type mice, reinforcing a role for OPN in modulating systemic metabolic activity and confirming results of other researchers related to the role of OPN in modulating obesity (100, 101).

Collectively, impressive data exists supporting a role for OPN in affecting systemic metabolic activity, although further studies are warranted to determine if the RGD region of OPN is involved.

BSP: BSP is more selective to mineralized tissues and most studies have focused on defining the mechanisms controlling BSP function in mineralized tissues. Data from studies using BSP-deficient mice reveal that BSP is a modulator of mineralization. The reader is refereed to several articles and references thereof for details on tooth/bone phenotype of BSP-deficient mice (7, 102–106). In brief, BSP-deficient mice have alteration in bone homeostasis and

mineralization (hypomineralized) and defects in the region of the periodontium, to include impairments in formation of cementum and surrounding alveolar bone, resulting in a disorganized periodontal ligament (PDL) region, malocclusion and exfoliation of teeth, similar to mice and humans with alkaline phosphatase mutations (30). To define the role of the RGD domain of BSP in controlling periodontal tissues, Nagasaki et al. generated BSP-KAE knock-in (KI) mice, substituting a non-function KAE (lysine-alanine-glutamic acid) sequence for the RGD. The results emphasized the importance of the RGD region for forming and maintaining the PDL region, but not for promoting mineralization (107).

To the surprise of Nagasaki et al. the BSP-RGD non-functionalized mice exhibited increased body weight and energy intake with age (after 13 weeks postnatal) vs. wild-type mice. Accordingly, weight of epididymal fat pad and size of white and brown adipocytes were increased (108).

Brief reviews of metabolic functions of other RGD containing proteins, insulin-like growth factor-binding proteins 1 and 2 (IGFBP-1, IGFBP-2), developmental endothelial locus-1 (DEL-1) and milk fat globule–EGF factor-8 (MFG-E8) are provided below, with references to reviews for more extensive reports on their various roles.

## Other RGD-containing proteins proposed to affect systemic metabolic activity

### IGFBP-1, 2

Insulin-like growth factor-binding proteins 1 and 2 (IGFBP 1 and 2) are members of a highly conserved family of six IGFBPs, numbered IGFBP1 through 6 in vertebrates, that modulate the actions of insulin growth factors (IGFs) and play vital roles in regulating several cellular processes (15, 17, 109, 110). The IGFs act as both endocrine hormones and autocrine/paracrine growth factors, binding to the IGFBPs or IGF-1 receptor. While the IGFBPs share about 50% homology with each other, each has specific structural features and plays distinct roles locally and systemically. In addition to the role in modulating circulating and local levels on IGF *via* IGF-IGFBP binding, IGFBPs also have activities independent of IGF binding. IGFBP-1 and 2 are the smallest in size, 25kDa and 31kDa respectively, and are the only IGFBPs that have RGD sequences. Interestingly, IGFBP1 and 2 are reported to serve as markers of autoimmune diseases such as Type 1 diabetes mellitus and rheumatoid arthritis (110).

For IGFBP 1 and 2, their RGD region allows them to influence cell adhesion, migration and signaling (18, 111, 112). Data from mouse studies suggest that the RGD motifs of both IGFBP1 and 2, signaling through specific integrins to include α5β1, affect energy metabolism (111, 112). Further studies by Haywood et al., using both *in vitro* and *in vivo* models (the latter, metabolic profiling of obese mice), evaluated whether the RGD domain of IGFBP1 could affect insulin sensitivity, insulin secretion and whole-body glucose regulation (16). Their results suggest that the RGD-integrin binding domain *via* cell signaling enhanced insulin sensitivity and secretion and administration of RGD synthetic peptide to obese mice could improve glucose clearance and insulin sensitivity. The authors conclude that the RGD domain of IGFBP-1 may hold promise as a therapeutic approach to insulin resistance. In another study, using IGFBP-2 transgenic mice (female), Reyer et al. showed that an IGFBP-2-RGD dependent mechanism was associated with impaired glucose clearance and regulation of GLUT4 translocation in muscle (18).

### DEL1 and MFG-E8

Developmental endothelial locus-1 (DEL-1) and milk fat globule–EGF factor-8 (MFG-E8) are structurally related proteins consisting of *N*-terminal EGF-like repeats and *C*-terminal discoidin I-like domains (20, 25). An RGD motif present in the second EGF-like repeat enables both proteins to interact with integrins such as αvβ3, αvβ5, although DEL-1 has also been shown to bind to non-RGD-binding integrins (αLβ2, αMβ2). DEL-1 and MFG-E8 generally behave as anti-inflammatory and pro-resolving proteins, in large part due to their capacity to promote efferocytosis (26, 113). Additionally, DEL-1 inhibits inflammation by regulating neutrophil recruitment and T regulatory cell function (27, 114). DEL-1 and MFG-E8 have been shown to promote tissue healing (21, 28) and bone homeostasis through effects on both osteoblasts and osteoclasts (22, 28, 29, 115, 116). In this regard, local administration of recombinant DEL-1 or MFG-E8 in the gingiva of non-human primates inhibits periodontal bone loss (29, 117). Despite sharing several functions, DEL-1 and MFG-E8 mediate non-redundant roles in *in vivo* experimental models, presumably owing to their expression in different tissues, often by different cell types, and/or their regulation by distinct transcription factors (20, 26, 113, 118). It is also possible that these "cousin" proteins may be involved in metabolic regulation. MFG-E8 was reported to enhance the uptake of fatty acids by adipocytes and this function required an intact RGD motif since the effect was mediated by MFG-E8 interaction with $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins (23, 24). In the same study, MFG-E8-deficient mice were protected from diet-induced obesity (23). It is currently uncertain whether DEL-1 shares a similar metabolic function, although its ability to interact with RGD-binding integrins warrants relevant investigation. Interestingly in that regard, *EDIL3*, the gene encoding DEL-1, has been associated with susceptibility to childhood obesity (119) and is overexpressed in diet-induced obesity in mice as well as in obese humans (120).

Coupled with the above evidence providing a role for the RGD region of proteins being involved in modulating energy metabolism are studies highlighting that RGD-binding integrins

may serve as therapeutic targets for controlling metabolic activity and diseases, such as diabetic retinopathy (121), sepsis (122) and inflammatory disorders (123).

### Non-RGD containing proteins, associated with mineralized tissues, purported to influence systemic metabolic activity:

While this perspective highlights the RGD motif of proteins and their role in controlling metabolic activity, the fact that many genes/proteins associated with mineralized tissues have been reported to affect systemic metabolic activity provides additional support for the concept that mineralized tissues, through direct or indirect mechanisms, are important players in controlling whole-body homeostasis. Table 2 provides a brief overview of some of these factors, with additional references within these tables (31–49), for more detailed information.

## Discussion/Conclusion

This perspective provides data from studies over decades clearly showing that factors secreted by mineralized tissues as well as other tissues influence bone metabolic activity and also systemic metabolic activity. Yet, there remain missing pieces of the puzzle related to the mechanistic aspects for the genes and associated proteins affecting bone/systemic metabolic activity. Featured in this perspective is the need to consider the role of RGD-associated proteins in monitoring metabolic activity locally and systemically, with more answers to be forthcoming as investigations continue along this line of research. Such studies are important to better understand whether therapeutics targeting specific proteins and/or the specific RGD region may be attractive treatments for controlling obesity and associated diseases.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## Author contributions

KN, OG, GH, and MS, contributed to this perspective from discussions on the outline of topics to be included, to working on and reviewing the text and tables, to finalizing the manuscript for peer review. All authors agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

## Funding

KN and MS were supported by NIAMS/NIH Intramural Program. OG was supported by NIDDK/NIH Intramural Program. GH is supported by NIDCR/NIH Grants DE026152 and DE028561.

## Acknowledgments

The authors wish to acknowledge the numerous researchers, over many decades, for their important contributions toward advancing our understanding of the role of mineralized tissues in modulating local and systemic metabolic activity.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## References

1. Wei J, Ferron M, Clarke CJ, Hannun YA, Jiang H, Blaner WS, et al. Bone-specific insulin resistance disrupts whole-body glucose homeostasis via decreased osteocalcin activation. *J Clin Invest.* (2014) 124:1–13. doi: 10.1172/JCI72323

2. Confavreux CB, Levine RL, Karsenty G, A. paradigm of integrative physiology, the crosstalk between bone and energy metabolisms. *Mol Cell Endocrinol.* (2009) 310:21–9. doi: 10.1016/j.mce.2009.04.004

3. Goettsch C, Strzelecka-Kiliszek A, Bessueille L, Quillard T, Mechtouff L, Pikula S, et al. TNAP as a therapeutic target for cardiovascular calcification: a discussion of its pleiotropic functions in the body. *Cardiovasc Res.* (2022) 118:84–96. doi: 10.1093/cvr/cvaa299

4. Fulzele K, Riddle RC, DiGirolamo DJ, Cao X, Wan C, Chen D, et al. Insulin receptor signaling in osteoblasts regulates postnatal bone acquisition and body composition. *Cell.* (2010) 142:309–19. doi: 10.1016/j.cell.2010.06.002

5. Bellahcène A, Castronovo V, Ogbureke KU, Fisher LW, Fedarko NS. Small integrin-binding ligand N-linked glycoproteins (SIBLINGs): multifunctional proteins in cancer. *Nat Rev Cancer.* (2008) 8:212–26. doi: 10.1038/nrc2345

6. Bellahcène A, Bonjean K, Fohr B, Fedarko NS, Robey FA, Young MF, et al. Bone sialoprotein mediates human endothelial cell attachment and migration and promotes angiogenesis. *Circ Res.* (2000) 86:885–91. doi: 10.1161/01.RES.86.8.885

7. Foster BL, Ao M, Willoughby C, Soenjaya Y, Holm E, Lukashova L, et al. Mineralization defects in cementum and craniofacial bone from loss of bone sialoprotein. *Bone.* (2015) 78:150–64. doi: 10.1016/j.bone.2015.05.007

8. Wu K, Feng J, Lyu F, Xing F, Sharma S, Liu Y, et al. Exosomal miR-19a and IBSP cooperate to induce osteolytic bone metastasis of estrogen receptor-positive breast cancer. *Nat Commun.* (2021) 12:5196. doi: 10.1038/s41467-021-25473-y

9. Chen Y, Qin Y, Dai M, Liu L, Ni Y, Sun Q, et al. IBSP, a potential recurrence biomarker, promotes the progression of colorectal cancer via Fyn/β-catenin signaling pathway. *Cancer Med.* (2021) 10:4030–45. doi: 10.1002/cam4.3959

10. Foster BL, Ao M, Salmon CR, Chavez MB, Kolli TN, Tran AB, et al. Osteopontin regulates dentin and alveolar bone development and mineralization. *Bone.* (2018) 107:196–207. doi: 10.1016/j.bone.2017.12.004

11. Nomiyama T, Perez-Tilve D, Ogawa D, Gizard F, Zhao Y, Heywood EB, et al. Osteopontin mediates obesity-induced adipose tissue macrophage infiltration and insulin resistance in mice. *J Clin Invest.* (2007) 117:2877–88. doi: 10.1172/JCI31986

12. Kiefer FW, Zeyda M, Gollinger K, Pfau B, Neuhofer A, Weichhart T, et al. Neutralization of osteopontin inhibits obesity-induced inflammation and insulin resistance. *Diabetes.* (2010) 59:935–46. doi: 10.2337/db09-0404

13. Dai B, Xu J, Li X, Huang L, Hopkins C, Wang H, et al. Macrophages in epididymal adipose tissue secrete osteopontin to regulate bone homeostasis. *Nat Commun.* (2022) 13:427. doi: 10.1038/s41467-021-27683-w

14. Zhao H, Chen Q, Alam A, Cui J, Suen KC, Soo AP, et al. The role of osteopontin in the progression of solid organ tumour. *Cell Death Dis.* (2018) 9:356. doi: 10.1038/s41419-018-0391-6

15. Hoeflich A, Russo VC. Physiology and pathophysiology of IGFBP-1 and IGFBP-2 - consensus and dissent on metabolic control and malignant potential. *Best Pract Res Clin Endocrinol Metab.* (2015) 29:685–700. doi: 10.1016/j.beem.2015.07.002

16. Haywood NJ, Cordell PA, Tang KY, Makova N, Yuldasheva NY, Imrie H, et al. Insulin-like growth factor binding protein 1 could improve glucose regulation and insulin sensitivity through its RGD domain. *Diabetes.* (2017) 66:287–99. doi: 10.2337/db16-0997

17. Li T, Forbes ME, Fuller GN Li J, Yang X, Zhang W. IGFBP2: integrative hub of developmental and oncogenic signaling network. *Oncogene.* (2020) 39:2243–57. doi: 10.1038/s41388-020-1154-2

18. Reyer A, Schindler N, Ohde D, Walz C, Kunze M, Tuchscherer A, et al. The RGD sequence present in IGFBP-2 is required for reduced glucose clearance after oral glucose administration in female transgenic mice. *Am J Physiol Endocrinol Metab.* (2015) 309:E409–17. doi: 10.1152/ajpendo.00168.2015

19. Zhang X, Gu HF, Frystyk J, Efendic S, Brismar K, Thorell A. Analyses of IGFBP2 DNA methylation and mRNA expression in visceral and subcutaneous adipose tissues of obese subjects. *Growth Horm IGF Res.* (2019) 45:31–6. doi: 10.1016/j.ghir.2019.03.002

20. Aziz M, Jacob A, Matsuda A, Wang P. Review: milk fat globule-EGF factor 8 expression, function and plausible signal transduction in resolving inflammation. *Apoptosis.* (2011) 16:1077–86. doi: 10.1007/s10495–011–0630–0

21. Bu HF, Zuo XL, Wang X, Ensslin MA, Koti V, Hsueh W, et al. Milk fat globule-EGF factor 8/lactadherin plays a crucial role in maintenance and repair of murine intestinal epithelium. *J Clin Invest.* (2007) 117:3673–83. doi: 10.1172/JCI31841

22. Abe T, Shin J, Hosur K, Udey MC, Chavakis T, Hajishengallis G. Regulation of osteoclast homeostasis and inflammatory bone loss by MFG-E8. *J Immunol.* (2014) 193:1383–91. doi: 10.4049/jimmunol.1400970

23. Khalifeh-Soltani A, McKleroy W, Sakuma S, Cheung YY, Tharp K, Qiu Y, et al. Mfge8 promotes obesity by mediating the uptake of dietary fats and serum fatty acids. *Nat Med.* (2014) 20:175–83. doi: 10.1038/nm.3450

24. Ren Y, Liu W, Zhang J, Bi J, Fan M, Lv Y, et al. MFG-E8 maintains cellular homeostasis by suppressing endoplasmic reticulum stress in pancreatic exocrine acinar cells. *Front Cell Dev Biol.* (2021) 9:803876. doi: 10.3389/fcell.2021.803876

25. Hajishengallis G, Chavakis T. DEL-1-regulated immune plasticity and inflammatory disorders. *Trends Mol Med.* (2019) 25:444–59. doi: 10.1016/j.molmed.2019.02.010

26. Kourtzelis I, Li X, Mitroulis I, Grosser D, Kajikawa T, Wang B, et al. DEL-1 promotes macrophage efferocytosis and clearance of inflammation. *Nat Immunol.* (2019) 20:40–9. doi: 10.1038/s41590-018-0249-1

27. Li X, Colamatteo A, Kalafati L, Kajikawa T, Wang H, Lim J-H, et al. The DEL-1/β3 integrin axis promotes regulatory T cell responses during inflammation resolution. *J Clin Invest.* (2020) 130:6261–77. doi: 10.1172/JCI137530

28. Yuh DY, Maekawa T, Li X, Kajikawa T, Bdeir K, Chavakis T, et al. The secreted protein DEL-1 activates a β3 integrin-FAK-ERK1/2-RUNX2 pathway and promotes osteogenic differentiation and bone regeneration. *J Biol Chem.* (2020) 295:7261–73. doi: 10.1074/jbc.RA120.013024

29. Shin J, Maekawa T, Abe T, Hajishengallis E, Hosur K, Pyaram K, et al. DEL-1 restrains osteoclastogenesis and inhibits inflammatory bone loss in nonhuman primates. *Sci Transl Med.* (2015) 7:307ra155. doi: 10.1126/scitranslmed.aac5380

30. Millán JL, Whyte MP. Alkaline phosphatase and hypophosphatasia. *Calcif Tissue Int.* (2016) 98:398–416. doi: 10.1007/s00223-015-0079-1

31. Briolay A, Bessueille L, Magne D. TNAP: a new multitask enzyme in energy metabolism. *Int J Mol Sci.* (2021) 22:10470. doi: 10.3390/ijms221910470

32. Sun Y, Rahbani JF, Jedrychowski MP, Riley CL, Vidoni S, Bogoslavski D, et al. Mitochondrial TNAP controls thermogenesis by hydrolysis of phosphocreatine. *Nature.* (2021) 593:580–5. doi: 10.1038/s41586-021-03533-z

33. Bessueille L, Briolay A, Como J, Mebarek S, Mansouri C, Gleizes M, et al. Tissue-nonspecific alkaline phosphatase is an anti-inflammatory nucleotidase. *Bone.* (2020) 133:115262. doi: 10.1016/j.bone.2020.115262

34. Krishnamurthy VR, Baird BC, Wei G, Greene T, Raphael K, Beddhu S. Associations of serum alkaline phosphatase with metabolic syndrome and mortality. *Am J Med.* (2011) 124:566.e1–7. doi: 10.1016/j.amjmed.2010.11.030

35. Graser S, Liedtke D, Jakob F, TNAP. as a new player in chronic inflammatory conditions and metabolism. *Int J Mol Sci.* (2021) 22:919. doi: 10.3390/ijms22020919

36. Bacchetta J, Bardet C, Prié D. Physiology of FGF23 and overview of genetic diseases associated with renal phosphate wasting. *Metabolism.* (2020) 103S:153865. doi: 10.1016/j.metabol.2019.01.006

37. Bhattacharyya N, Chong WH, Gafni RI, Collins MT. Fibroblast growth factor 23: state of the field and future directions. *Trends Endocrinol Metab.* (2012) 23:610–8. doi: 10.1016/j.tem.2012.07.002

38. Minisola S, Peacock M, Fukumoto S, Cipriani C, Pepe J, Tella SH, et al. Tumour-induced osteomalacia. *Nat Rev Dis Primers.* (2017) 3:17044. doi: 10.1038/nrdp.2017.44

39. Wei J, Karsenty G. An overview of the metabolic functions of osteocalcin. *Curr Osteoporos Rep.* (2015) 13:180–5. doi: 10.1007/s11914-015-0267-y

40. Ferron M, McKee MD, Levine RL, Ducy P, Karsenty G. Intermittent injections of osteocalcin improve glucose metabolism and prevent type 2 diabetes in mice. *Bone.* (2012) 50:568–75. doi: 10.1016/j.bone.2011.04.017

41. Ferron M, Hinoi E, Karsenty G, Ducy P. Osteocalcin differentially regulates beta cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. *Proc Natl Acad Sci U S A.* (2008) 105:5266–70. doi: 10.1073/pnas.0711119105

42. Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, et al. Increased bone formation in osteocalcin-deficient mice. *Nature.* (1996) 382:448–52. doi: 10.1038/382448a0

43. Suchacki KJ, Morton NM, Vary C, Huesa C, Yadav MC, Thomas BJ, et al. PHOSPHO1 is a skeletal regulator of insulin resistance and obesity. *BMC Biol.* (2020) 18:149. doi: 10.1186/s12915-020-00880-7

44. Zweifler LE, Ao M, Yadav M, Kuss P, Narisawa S, Kolli TN, et al. Role of PHOSPHO1 in periodontal development and function. *J Dent Res.* (2016) 95:742–51. doi: 10.1177/0022034516640246

45. Houston B, Stewart AJ, Farquharson C. PHOSPHO1-A novel phosphatase specifically expressed at sites of mineralisation in bone and cartilage. *Bone.* (2004) 34:629–37. doi: 10.1016/j.bone.2003.12.023

46. McKee MD, Yadav MC, Foster BL, Somerman MJ, Farquharson C, Millán JL. Compounded PHOSPHO1/ALPL deficiencies reduce dentin mineralization. *J Dent Res.* (2013) 92:721–7. doi: 10.1177/0022034513490958

47. Rowe PS. Regulation of bone-renal mineral and energy metabolism: the PHEX, FGF23, DMP1, MEPE ASARM pathway. *Crit Rev Eukaryot Gene Expr.* (2012) 22:61–86. doi: 10.1615/CritRevEukarGeneExpr.v22.i1.50

48. Rowe PS, de Zoysa PA, Dong R, Wang HR, White KE, Econs MJ, et al. MEPE, a new gene expressed in bone marrow and tumors causing osteomalacia. *Genomics.* (2000) 67:54–68. doi: 10.1006/geno.2000.6235

49. Salmon B, Bardet C, Khaddam M, Naji J, Coyac BR, Baroukh B, et al. MEPE-derived ASARM peptide inhibits odontogenic differentiation of dental pulp stem cells and impairs mineralization in tooth models of X-linked hypophosphatemia. *PLoS ONE.* (2013) 8:e56749. doi: 10.1371/journal.pone.0056749

50. Kim KS, Seeley RJ, Sandoval DA. Signalling from the periphery to the brain that regulates energy homeostasis. *Nat Rev Neurosci.* (2018) 19:185–96. doi: 10.1038/nrn.2018.8

51. Jais A, Brüning JC. Arcuate nucleus-dependent regulation of metabolism-pathways to obesity and diabetes mellitus. *Endocr Rev.* (2022) 43:314–28. doi: 10.1210/endrev/bnab025

52. Priest C, Tontonoz P. Inter-organ cross-talk in metabolic syndrome. *Nat Metab.* (2019) 1:1177–88. doi: 10.1038/s42255-019-0145-5

53. Funcke JB, Scherer PE. Beyond adiponectin and leptin: adipose tissue-derived mediators of inter-organ communication. *J Lipid Res.* (2019) 60:1648–84. doi: 10.1194/jlr.R094060

54. Yang Q, Vijayakumar A, Kahn BB. Metabolites as regulators of insulin sensitivity and metabolism. *Nat Rev Mol Cell Biol.* (2018) 19:654–72. doi: 10.1038/s41580-018-0044-8

55. Liu S, Alexander RK, Lee CH. Lipid metabolites as metabolic messengers in inter-organ communication. *Trends Endocrinol Metab.* (2014) 25:356–63. doi: 10.1016/j.tem.2014.05.002

56. Crewe C, Scherer PE. Intercellular and interorgan crosstalk through adipocyte extracellular vesicles. *Rev Endocr Metab Disord.* (2022) 23:61–9. doi: 10.1007/s11154-020-09625-x

57. Romero A, Eckel J. Organ crosstalk and the modulation of insulin signaling. *Cells.* (2021) 10:2082. doi: 10.3390/cells10082082

58. Rosen ED, Spiegelman BM. What we talk about when we talk about fat. *Cell.* (2014) 156:20–44. doi: 10.1016/j.cell.2013.12.012

59. Sakers A, De Siqueira MK, Seale P, Villanueva CJ. Adipose-tissue plasticity in health and disease. *Cell.* (2022) 185:419–46. doi: 10.1016/j.cell.2021.12.016

60. Czech MP. Mechanisms of insulin resistance related to white, beige, and brown adipocytes. *Mol Metab.* (2020) 34:27–42. doi: 10.1016/j.molmet.2019.12.014

61. Mann JP, Savage DB. What lipodystrophies teach us about the metabolic syndrome. *J Clin Invest.* (2019) 129:4009–21. doi: 10.1172/JCI129190

62. Ouchi N, Parker JL, Lugus JJ, Walsh K. Adipokines in inflammation and metabolic disease. *Nat Rev Immunol.* (2011) 11:85–97. doi: 10.1038/nri2921

63. Lee YS, Olefsky J. Chronic tissue inflammation and metabolic disease. *Genes Dev.* (2021) 35:307–28. doi: 10.1101/gad.346312.120

64. Suzuki A, Minamide M, Iwaya C, Ogata K, Iwata J. Role of metabolism in bone development and homeostasis. *Int J Mol Sci.* (2020) 21:8992. doi: 10.3390/ijms21238992

65. Karsenty G, Khosla S. The crosstalk between bone remodeling and energy metabolism: a translational perspective. *Cell Metab.* (2022) 34:805–17. doi: 10.1016/j.cmet.2022.04.010

66. Wee NK, Kulkarni RN, Horsnell H, Baldock PA. The brain in bone and fuel metabolism. *Bone.* (2016) 82:56–63. doi: 10.1016/j.bone.2015.10.020

67. Parimisetty A, Dorsemans AC, Awada R, Ravanan P, Diotel N. Lefebvre d'Hellencourt C. Secret talk between adipose tissue and central nervous system via secreted factors-an emerging frontier in the neurodegenerative research. *J Neuroinflammation.* (2016) 13:67. doi: 10.1186/s12974-016-0530-x

68. Thommesen L, Stunes AK, Monjo M, Grøsvik K, Tamburstuen MV, Kjøbli E, et al. Expression and regulation of resistin in osteoblasts and osteoclasts indicate a role in bone metabolism. *J Cell Biochem.* (2006) 99:824–34. doi: 10.1002/jcb.20915

69. Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, et al. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell.* (2000) 100:197–207. doi: 10.1016/S0092-8674(00)81558-5

70. Lewis JW, Edwards JR, Naylor AJ, McGettrick HM. Adiponectin signalling in bone homeostasis, with age and in disease. *Bone Res.* (2021) 9:1. doi: 10.1038/s41413-020-00122-0

71. Horowitz MC, Berry R, Holtrup B, Sebo Z, Nelson T, Fretz JA, et al. Bone marrow adipocytes. *Adipocyte.* (2017) 6:193–204. doi: 10.1080/21623945.2017.1367881

72. Pachón-Peña G, Bredella MA. Bone marrow adipose tissue in metabolic health. *Trends Endocrinol Metab.* (2022) 33:401–8. doi: 10.1016/j.tem.2022.03.003

73. Li J, Liu X, Zuo B, Zhang L. The role of bone marrow microenvironment in governing the balance between osteoblastogenesis and adipogenesis. *Aging Dis.* (2016) 7:514–25. doi: 10.14336/AD.2015.1206

74. Ning K, Liu S, Yang B, Wang R, Man G, Wang DE, et al. Update on the effects of energy metabolism in bone marrow mesenchymal stem cells differentiation. *Mol Metab.* (2022) 58:101450. doi: 10.1016/j.molmet.2022.101450

75. Ruoslahti E, RGD. and other recognition sequences for integrins. *Annu Rev Cell Dev Biol.* (1996) 12:697–715. doi: 10.1146/annurev.cellbio.12.1.697

76. Ludwig BS, Kessler H, Kossatz S, Reuning U. RGD-binding integrins revisited: how recently discovered functions and novel synthetic ligands (Re-)shape an ever-evolving field. *Cancers (Basel).* (2021) 13:1711. doi: 10.3390/cancers13071711

77. Nieberler M, Reuning U, Reichart F, Notni J, Wester HJ, Schwaiger M, et al. Exploring the role of RGD-recognizing integrins in cancer. *Cancers (Basel).* (2017) 9:116. doi: 10.3390/cancers9090116

78. Zhu J, Springer TA. Complete integrin headpiece opening in eight steps. *J Cell Biol.* (2013) 201:1053–68. eng. doi: 10.1083/jcb.201212037

79. Harburger DS, Calderwood DA. Integrin signalling at a glance. *J Cell Sci.* (2009) 122:159–63. doi: 10.1242/jcs.018093

80. Fisher LW, Torchia DA, Fohr B, Young MF, Fedarko NS. Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. *Biochem Biophys Res Commun.* (2001) 280:460–5. doi: 10.1006/bbrc.2000.4146

81. Fisher LW, Fedarko NS. Six genes expressed in bones and teeth encode the current members of the SIBLING family of proteins. *Connect Tissue Res.* (2003) 44 Suppl. 1:33–40. doi: 10.1080/03008200390152061

82. Fisher LW, Whitson SW, Avioli LV, Termine JD. Matrix sialoprotein of developing bone. *J Biol Chem.* (1983) 258:12723–7. doi: 10.1016/S0021-9258(17)44236-0

83. Somerman MJ, Sauk JJ, Foster RA, Norris K, Dickerson K, Argraves WS. Cell attachment activity of cementum: bone sialoprotein II identified in cementum. *J Periodontal Res.* (1991) 26:10–6. doi: 10.1111/j.1600-0765.1991.tb01620.x

84. Somerman MJ, Prince CW, Sauk JJ, Foster RA, Butler WT. Mechanism of fibroblast attachment to bone extracellular matrix: role of a 44 kilodalton bone phosphoprotein. *J Bone Miner Res.* (1987) 2:259–65. doi: 10.1002/jbmr.5650020313

85. Oldberg A, Franzén A, Heinegård D. Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. *Proc Natl Acad Sci U S A.* (1986) 83:8819–23. doi: 10.1073/pnas.83.23.8819

86. Hunter GK, Goldberg HA. Nucleation of hydroxyapatite by bone sialoprotein. *Proc Natl Acad Sci U S A.* (1993) 90:8562–5. doi: 10.1073/pnas.90.18.8562

87. Fisher LW, McBride OW, Termine JD, Young MF. Human bone sialoprotein. Deduced protein sequence and chromosomal localization. *J Biol Chem.* (1990) 265:2347–51. doi: 10.1016/S0021-9258(19)39982-X

88. Hunter GK, Hauschka PV, Poole AR, Rosenberg LC, Goldberg HA. Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. *Biochem J.* (1996) 317:59–64. doi: 10.1042/bj3170059

89. Gericke A, Qin C, Spevak L, Fujimoto Y, Butler WT, Sørensen ES, et al. Importance of phosphorylation for osteopontin regulation of biomineralization. *Calcif Tissue Int.* (2005) 77:45–54. doi: 10.1007/s00223-004-1288-1

90. Sørensen ES, Højrup P, Petersen TE. Posttranslational modifications of bovine osteopontin: identification of twenty-eight phosphorylation and three O-glycosylation sites. *Protein Sci.* (1995) 4:2040–9. doi: 10.1002/pro.5560041009

91. Harmey D, Johnson KA, Zelken J, Camacho NP, Hoylaerts MF, Noda M, et al. Elevated skeletal osteopontin levels contribute to the hypophosphatasia phenotype in Akp2(-/-) mice. *J Bone Miner Res.* (2006) 21:1377–86. doi: 10.1359/jbmr.060619

92. Si J, Wang C, Zhang D, Wang B, Zhou Y. Osteopontin in bone metabolism and bone diseases. *Med Sci Monit.* (2020) 26:e919159. doi: 10.12659/MSM.919159

93. Kahles F, Findeisen HM, Bruemmer D. Osteopontin: a novel regulator at the cross roads of inflammation, obesity and diabetes. *Mol Metab.* (2014) 3:384–93. doi: 10.1016/j.molmet.2014.03.004

94. Lancha A, Rodríguez A, Catalán V, Becerril S, Sáinz N, Ramírez B, et al. Osteopontin deletion prevents the development of obesity and hepatic steatosis via impaired adipose tissue matrix remodeling and reduced inflammation and fibrosis in adipose tissue and liver in mice. *PLoS ONE.* (2014) 9:e98398. doi: 10.1371/journal.pone.0098398

95. Denhardt DT, Noda M, O'Regan AW, Pavlin D, Berman JS. Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. *J Clin Invest.* (2001) 107:1055–61. doi: 10.1172/JCI12980

96. Icer MA, Gezmen-Karadag M. The multiple functions and mechanisms of osteopontin. *Clin Biochem.* (2018) 59:17–24. doi: 10.1016/j.clinbiochem.2018.07.003

97. Nuñez-Garcia M, Gomez-Santos B, Buqué X, García-Rodriguez JL, Romero MR, Marin JJG, et al. Osteopontin regulates the cross-talk between phosphatidylcholine and cholesterol metabolism in mouse liver. *J Lipid Res.* (2017) 58:1903–15. doi: 10.1194/jlr.M078980

98. Gómez-Santos B, Saenz de. Urturi D, Nuñez-García M, Gonzalez-Romero F, Buque X, Aurrekoetxea I, et al. Liver osteopontin is required to prevent the

progression of age-related nonalcoholic fatty liver disease. *Aging Cell.* (2020) 19:e13183. doi: 10.1111/acel.13183

99. Chen Q, Shou P, Zhang L, Xu C, Zheng C, Han Y, et al. An osteopontin-integrin interaction plays a critical role in directing adipogenesis and osteogenesis by mesenchymal stem cells. *Stem Cells.* (2014) 32:327–37. doi: 10.1002/stem.1567

100. Kiefer FW, Neschen S, Pfau B, Legerer B, Neuhofer A, Kahle M, et al. Osteopontin deficiency protects against obesity-induced hepatic steatosis and attenuates glucose production in mice. *Diabetologia.* (2011) 54:2132–42. doi: 10.1007/s00125-011-2170-0

101. Chapman J, Miles PD, Ofrecio JM, Neels JG Yu JG, Resnik JL, et al. Osteopontin is required for the early onset of high fat diet-induced insulin resistance in mice. *PLoS ONE.* (2010) 5:e13959. doi: 10.1371/journal.pone.0013959

102. Bouleftour W, Boudiffa M, Wade-Gueye NM, Bouët G, Cardelli M, Laroche N, et al. Skeletal development of mice lacking bone sialoprotein (BSP)–impairment of long bone growth and progressive establishment of high trabecular bone mass. *PLoS ONE.* (2014) 9:e95144. doi: 10.1371/journal.pone.0095144

103. Malaval L, Wade-Guéye NM, Boudiffa M, Fei J, Zirngibl R, Chen F, et al. Bone sialoprotein plays a functional role in bone formation and osteoclastogenesis. *J Exp Med.* (2008) 205:1145–53. doi: 10.1084/jem.20071294

104. Foster BL, Soenjaya Y, Nociti FH, Holm E, Zerfas PM, Wimer HF, et al. Deficiency in acellular cementum and periodontal attachment in bsp null mice. *J Dent Res.* (2013) 92:166–72. doi: 10.1177/0022034512469026

105. Bouleftour W, Bouet G, Granito RN, Thomas M, Linossier MT, Vanden-Bossche A, et al. Blocking the expression of both bone sialoprotein (BSP) and osteopontin (OPN) impairs the anabolic action of PTH in mouse calvaria bone. *J Cell Physiol.* (2015) 230:568–77. doi: 10.1002/jcp.24772

106. Bouleftour W, Juignet L, Verdière L, Machuca-Gayet I, Thomas M, Laroche N, et al. Deletion of OPN in BSP knockout mice does not correct bone hypomineralization but results in high bone turnover. *Bone.* (2019) 120:411–22. doi: 10.1016/j.bone.2018.12.001

107. Nagasaki K, Chavez MB, Nagasaki A, Taylor JM, Tan MH, Ma M, et al. The bone sialoprotein RGD domain modulates and maintains periodontal development. *J Dent Res.* (2022) 101:220345221100794. doi: 10.1177/00220345221100794

108. Taylor J, Nagasaki A, Kear B, Brock S, Ma Y, Gavrilova O, et al. The BSP-RGD motif affects PDL organization and age-related obesity 2021 IADR/AADR/CADR general session. Bethesda, MD: Archives IADR.

109. Allard JB, Duan C. IGF-binding proteins: why do they exist and why are there so many? *Front Endocrinol (Lausanne).* (2018) 9:117. doi: 10.3389/fendo.2018.00117

110. Ding H, Wu T. Insulin-like growth factor binding proteins in autoimmune diseases. *Front Endocrinol (Lausanne).* (2018) 9:499. doi: 10.3389/fendo.2018.00499

111. Jones JI, Gockerman A, Busby WH, Wright G, Clemmons DR. Insulin-like growth factor binding protein 1 stimulates cell migration and binds to the alpha 5 beta 1 integrin by means of its Arg-Gly-Asp sequence. *Proc Natl Acad Sci U S A.* (1993) 90:10553–7. doi: 10.1073/pnas.90.22.10553

112. Feng N, Zhang Z, Wang Z, Zheng H, Qu F, He X, et al. Insulin-like growth factor binding protein-2 promotes adhesion of endothelial progenitor cells to endothelial cells via integrin α5β1. *J Mol Neurosci.* (2015) 57:426–34. doi: 10.1007/s12031-015-0589-3

113. Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, Uchiyama Y, et al. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science.* (2004) 304:1147–50. doi: 10.1126/science.1094359

114. Eskan MA, Jotwani R, Abe T, Chmelar J, Lim JH, Liang S, et al. The leukocyte integrin antagonist Del-1 inhibits IL-17-mediated inflammatory bone loss. *Nat Immunol.* (2012) 13:465–73. doi: 10.1096/fj.201701238R

115. Michalski MN, Seydel AL, Siismets EM, Zweifler LE, Koh AJ, Sinder BP, et al. Inflammatory bone loss associated with MFG-E8 deficiency is rescued by teriparatide. *FASEB J.* (2018) 32:3730–41.

116. Albus E, Sinningen K, Winzer M, Thiele S, Baschant U, Hannemann A, et al. Milk fat globule-epidermal growth factor 8 (MFG-E8) is a novel anti-inflammatory factor in rheumatoid arthritis in mice and humans. *J Bone Miner Res.* (2016) 31:596–605. doi: 10.1002/jbmr.2721

117. Kajikawa T, Meshikhes F, Maekawa T, Hajishengallis E, Hosur KB, Abe T, et al. Milk fat globule epidermal growth factor 8 inhibits periodontitis in non-human primates and its gingival crevicular fluid levels can differentiate periodontal health from disease in humans. *J Clin Periodontol.* (2017) 44:472–83. doi: 10.1111/jcpe.12707

118. Maekawa T, Hosur K, Abe T, Kantarci A, Ziogas A, Wang B, et al. Antagonistic effects of IL-17 and D-resolvins on endothelial Del-1 expression through a GSK-3beta-C/EBPbeta pathway. *Nat Commun.* (2015) 6:8272. doi: 10.1038/ncomms9272

119. Glessner JT, Bradfield JP, Wang K, Takahashi N, Zhang H, Sleiman PM, et al. A genome-wide study reveals copy number variants exclusive to childhood obesity cases. *Am J Hum Genet.* (2010) 87:661–6. doi: 10.1016/j.ajhg.2010.09.014

120. Cobb LP, Siamakpour-Reihani S, Zhang D, Qin X, Owzar K, Zhou C, et al. Obesity and altered angiogenic-related gene expression in endometrial cancer. *Gynecol Oncol.* (2021) 163:320–6. doi: 10.1016/j.ygyno.2021.08.010

121. Van Hove I, Hu TT, Beets K, Van Bergen T, Etienne I, Stitt AW, et al. Targeting RGD-binding integrins as an integrative therapy for diabetic retinopathy and neovascular age-related macular degeneration. *Prog Retin Eye Res.* (2021) 85:100966. doi: 10.1016/j.preteyeres.2021.100966

122. Nader D, Curley GF, Kerrigan SW, A. new perspective in sepsis treatment: could RGD-dependent integrins be novel targets? *Drug Discov Today.* (2020) 25:2317–25. doi: 10.1016/j.drudis.2020.09.038

123. Slack RJ, Macdonald SJF, Roper JA, Jenkins RG, Hatley RJD. Emerging therapeutic opportunities for integrin inhibitors. *Nat Rev Drug Discov.* (2022) 21:60–78. doi: 10.1038/s41573-021-00284-4

frontiers | Frontiers in Dental Medicine

TYPE Mini Review
PUBLISHED 11 August 2022
DOI 10.3389/fdmed.2022.985558



OPEN ACCESS

EDITED BY
Francisco Nociti,
Universidade Estadual de Campinas, Brazil

REVIEWED BY
Brenda P. Gomes,
Campinas State University, Brazil

*CORRESPONDENCE
Hajime Sasaki
hajimes@umich.edu
Philip Stashenko
pstash@bu.edu

†These authors share corresponding authorship.

SPECIALTY SECTION
This article was submitted to Endodontics, a section of the journal Frontiers in Dental Medicine

RECEIVED 04 July 2022
ACCEPTED 28 July 2022
PUBLISHED 11 August 2022

CITATION
Tazawa K, Azuma Presse MM, Furusho H, Stashenko P and Sasaki H (2022) Revisiting the role of IL-1 signaling in the development of apical periodontitis.
Front. Dent. Med 3:985558.
doi: 10.3389/fdmed.2022.985558



# Revisiting the role of IL-1 signaling in the development of apical periodontitis

Kento Tazawa[1,2†], Mariane Maffei Azuma Presse[1], Hisako Furusho[3], Philip Stashenko[4*†] and Hajime Sasaki[1*]

[1]Department of Cariology, Restorative Sciences and Endodontics, University of Michigan School of Dentistry, Ann Arbor, MI, United States, [2]Department of Pulp Biology and Endodontics, Division of Oral Health Sciences, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University (TMDU), Tokyo, Japan, [3]Department of Oral and Maxillofacial Pathobiology, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan, [4]Department of Translational Dental Medicine and Department of Endodontics, Boston University Goldman School of Dental Medicine, Boston, MA, United States

Apical periodontitis (AP) develops as a result of an immune response to pulpal bacterial infection, and various cytokines are involved in the pathogenesis of AP, with Interleukin (IL)-1 being considered a key cytokine. The role of IL-1 in the pathogenesis of AP has been well studied. It is known that IL-1 expression in periapical lesions correlates closely with the development of AP. IL-1 is a potent bone-resorptive cytokine that induces osteoclast formation and activation. Hence, inhibiting its signaling with IL-1 receptor antagonist (IL-1RA) results in a reduction in periapical lesion size. On the other hand, IL-1 is also a central cytokine that combats bacterial infection by activating innate immune responses. Therefore, a complete loss of IL-1 signaling leads to a failure to limit bacterial dissemination and consequently exacerbates AP. *In vivo*, IL-1 expression is tightly regulated and its signaling is modulated to optimize the immune response. Obesity causes systemic low-grade chronic inflammation and increases the risk of cardiovascular, renal, and other disorders. In experimentally induced AP, obesity significantly increases periapical bone loss, albeit the underlying mechanism remains unclear. Recent technological innovations have enabled more comprehensive and detailed analyses than previously, leading to new insights into the role of IL-1RA in regulating IL-1 signaling, and modulating apical lesion progression in obesity. In this review, we provide a brief overview of the function of IL-1 in AP development, with special emphasis on the latest findings in normal weight and obese states.

KEYWORDS

obesity, diabetes, apical periodontitis, cytokines, interleukin-1 signaling, immune response

Abbreviations

AP, apical periodontitis; DM, diabetes mellitus; DIO, diet-induced obesity; IFN-γ, gamma interferon; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; IL-1R1, interleukin-1 receptor type 1; IL-17RA, interleukin-17 receptor type A; MyD88, myeloid differentiation factor 88; NK cell, natural killer cell; OAF, osteoclast-activating factor; RANKL, receptor activator of NF-κB ligand; $T_{reg}$ cell, regulatory T cell; Th cell, thelper cell; TNF, tumor necrosis factor

## Introduction

Apical periodontitis (AP) involves chronic inflammation and alveolar bone loss. Kakehashi et al. demonstrated for the first time that AP is caused by pulpal infection. Rats maintained in a conventional microbial environment developed pulp necrosis and periapical inflammation after pulp exposure; In a germ-free environment, the pulps remained vital without periapical bone destruction, and dentin bridges formed over the exposed pulp, demonstrating the capacity for tissue regeneration in the absence of infection (1).

In response to infection, complexly mixed immune cells migrate to the infected site. First neutrophils infiltrate, a followed by monocytes/macrophages, and subsequently by lymphocytes [T, B, and natural killer (NK) cells] (2, 3). These cells play critical roles in innate and adaptive immunity. Innate immunity comprises nonspecific responses that do not require prior sensitization to an antigen. Phagocytes are key to innate responses; neutrophils and macrophages engulf bacteria, and NK cells eliminate infected cells. Innate cells also produce inflammatory cytokines, which mediate immune and connective tissue cell activity (4–6). To eliminate pathogens and establish immune memory, the adaptive response activates antigen-specific CD4 + helper and CD8 + cytotoxic T cells, as well as B cells and plasma cells that produce antibodies (7, 8). The innate immune system lastly eliminate bacteria, apoptotic/dead cells, and debris. These responses are precisely regulated by the complex cytokine network.

Cytokines thus primarily protect the pulp and periodontal tissue from infection; however, cytokine-activated immune and inflammatory responses induce tissue destruction, particularly bone resorption (9, 10). Regarding bone resorption, Horton et al. firstly reported that immune cells can influence osteoclast activity in 1972. Osteoclast-activating factor (OAF), a powerful stimulator of osteoclastic bone resorption, was released from human peripheral blood leukocytes stimulated by the mitogen phytohemagglutinin, or by antigenic material present in human dental plaque (11). In 1985, OAF was subsequently purified to homogeneity and sequenced, and shown to be identical to interleukin-1-beta (IL-1β). It was later shown that *macrophage-derived IL-1 is a prominent mediator in developing bone destructive periapical lesions* (12–15). These and other basic studies on the interactions between the immune system and bone following pulpal infections have been important in establishing the field of osteoimmunology. These basic studies have provided a rationale of clinical research on IL-1/IL-1 signaling in AP and foundation for interpreting their outcomes (16–22).

Obesity is one of the most prevalent non-communicable diseases and predisposes to various disorders, including hypertension, type 2 diabetes mellitus (DM), dyslipidemia, and coronary heart disease (23, 24). The increased morbidity associated with obesity is a worldwide public health issue (25). Besides, obese people are more susceptible to infections than their non-obese counterparts as well to developing serious complications from common infections (26). AP is one of the most prevalent oral infectious diseases. In DM subjects, where obesity is the greatest risk factor, the success of root canal treatment is decreased, in teeth with AP (27, 28). Moreover, studies in the rodent diet-induced obesity (DIO) model have revealed that obesity promotes the progression and severity of experimental AP (29–31). However, the underlying mechanism(s) by which obesity alters the immune response in AP remain unclear.

As the background for future basic and clinical research, this mini review aims first to reaffirm the role of IL-1 signaling in the development of AP in the lean state, and then to provide new insights into the possible mechanisms underlying the expansion of periapical bone destruction associated with obesity, based on the latest experimental findings.

## IL-1 signaling is the central pathway in periapical lesion development

The IL-1 family comprises 11 cytokines: 7 pro-inflammatory mediators (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ), and 4 anti-inflammatory cytokines [IL-1 receptor antagonist (RA), IL-36RA IL-37, and IL-38] (32). Each family member binds to a specific primary receptor which combines with co-receptors to transduce pro-inflammatory or anti-inflammatory activity. The primary receptors include IL-1 receptor type 1 (IL-1R1), IL-1R2, IL-1R4, IL-1R5, and IL-1R6. The co-receptors include IL-1R3, IL-1R7, IL-1R8, IL-1R9, and IL-1R10 (32, 33). IL-1α, IL-1β, and IL-1RA are the primary members that regulate the progression of periapical lesions, and their roles have been well studied. In contrast, the role of the other family members in the development of AP has not been systematically evaluated.

IL-1α and IL-1β are encoded by *IL1A* and *IL1B* respectively in humans (34). Both isoforms bind to IL-1R1 and show similar biologic activities, including immune cell activation (33, 35). IL-1 is also closely involved in both bone formation (36) and resorption (12, 15). IL-1 inhibits nodule formation by osteoblasts in a dose-dependent manner (36). IL-1 strongly promotes osteoclast differentiation indirectly by inducing the expression of receptor activator of NF-κB ligand (RANKL; Tumor necrosis factor ligand superfamily member 11) in osteoblasts (37). IL-1 directly induces the fusion of mononuclear pre-fusion osteoclasts and enhances osteoclast function (resorption pit-forming activity) (38–40). Moreover, activation of NF-κB promoted by IL-1 prolongs osteoclast survival (41, 42). However, IL-1α and IL-1β differ in several ways. First, species differences are found in their expression in

periapical lesions. In rodent lesions, the predominant isoform is IL-1α rather than IL-1β (43, 44). In contrast, the protein level of IL-1β in human periapical exudate is double that of IL-1α (45). Furthermore, the bone resorption potency of IL-1β is 13-fold that of IL-1α in a rat assay system (10). Second, the expression level after root canal treatment is different. Following treatment, the level of IL-1β in the periapical exudates decreased, while the level of IL-1α increased. This suggests that IL-1α and IL-1β may play different biological roles in the healing process (45, 46). In this regard, a finding that bacteria-induced IL-1β and IL-1RI-myeloid differentiation factor 88 (MyD88) signaling are necessary and sufficient for efficient wound healing and tissue regeneration (47) is interesting. Third, the IL-1β cannot bind to IL-1R1 unless it is cleaved into its biologically-active mature form. Conversely, IL-1α precursor can bind to and activate the IL-1 receptor without proteolysis (48).

The expression level of IL-1 positively correlates to the extension of bone destruction and severity of AP. IL-1α mRNA and protein expression was identified in murine periapical lesions from the early stage of development, with increased levels found on day 7 after pulp infection (43, 44, 49). Higher levels of IL-1α and IL-1β were detected in human periapical lesions with severe inflammation than mild inflammation (50, 51). In periapical lesions, IL-1 is produced by various cells, including macrophages, fibroblasts, polymorphonuclear leukocytes, endothelial cells, osteoblasts, and osteoclasts in response to infection (44, 49). Among these cells, macrophages are the major source of IL-1. Macrophage-derived IL-1 plays a critical role in the periapical immunity. IL-1β and IL-1α are respectively 1000- and 75-fold more potent in stimulating bone resorption than TNFα or TNFβ (lymphotoxin) respectively *in vitro* (10). Besides, IL-1 neutralization significantly reduced bone resorptive activity in extracts from periapical tissue explants, whereas TNF-α neutralization had no effect (13, 15).

These studies focused on the bone-destructive effects of IL-1, but IL-1 also protects the host early after bacterial challenge. Antibody-mediated neutralization of both IL-1α and IL-1β leads to a failure to contain pulpal infection in male but not female mice, resulting in orofacial abscesses and sepsis (52). Ovariectomized mice also developed sepsis, but were protected by an estrogen implant. Accordingly, IL-1 signaling synergizes with estrogen signaling to prime phagocytic cells for enhanced anti-microbial activity resulting in infection localization. IL-1R1 deficient mice identically showed severe bone destruction and sepsis after pulpal infection (53, 54). Taken together, a severe deficiency of IL-1 signaling leads to poor infection control, dissemination of infection, and elevated bone destruction.

Subsequent studies using IL-1RA have confirmed the correlation between IL-1 and bone resorption. IL-1RA, produced by macrophages and monocytes (55), competitively blocks the action of IL-1. IL-1RA binds to IL-1R1 with equal or greater affinity than IL-1α and IL-1β but does not activate downstream signaling (34, 55, 56). IL-1RA has a significant impact by suppressing periapical lesion development. Stashenko et al. demonstrated a 14-day IL-1RA treatment inhibited lesion development by approximately 60% (57). Maintaining IL-1 and IL-1RA in balance prevents excess inflammation and bone destruction. Once this balance is upset, inflammation and tissue damage may deteriorate (58). To block IL-1-mediated bone resorption *ex vivo*, rat fetal long bones and mouse newborn calvariae require approximately 10-fold and 100–1000-fold IL-1RA to IL-1, respectively (59). In periapical lesions, the level of IL-1RA is more abundant than IL-1 (mean IL-1RA: IL-1β ratio = 128: 7). Interestingly, exudates from symptomatic human lesions contained a significantly lower ratio of IL-1RA to IL-1β than exudates from asymptomatic human lesions (22). Taken together, the local balance of IL-1 and IL-1RA is crucially important in the periapical lesion development.

## The cytokine network in periapical lesions centered on IL-1 signaling

Macrophages are major players involved in the cytokine network, and secrete various immunoregulatory mediators, including IL-1 (35, 60). TNF-α is another pro-inflammatory cytokine expressed by macrophages (61) and increased in periapical lesions (44, 49). TNF-α promotes IL-1 secretion from murine resident peritoneal macrophages *in vitro* (62) and increases osteoclastogenesis by upregulating RANKL (63, 64). However as noted above, TNF-α itself is not much bone resorptive as IL-1 isoforms, and TNF-α deficient mice exhibited similar periapical lesion size to wild-type controls (65).

The role of type-1 T-helper (Th1) cytokines [Gamma interferon (IFN-γ), IL-12, IL-18] and Th2 cytokines (IL-4, IL-6, IL-10) on periapical bone destruction has also been evaluated. IFN-γ, IL-12, and IL-18 potentiate pro-inflammatory signaling (66–68) and their expression is increased in periapical lesions (43, 69, 70). IFN-γ modulates macrophage-derived IL-1 expression, but its effect is not consistent. IFN-γ promotes secretion of IL-1 from LPS-stimulated human macrophages *in vitro* (71), whereas suppresses IL-1 in mouse RAW 264.7 macrophages (72). IL-12 induces Th1 cell development, and IL-18, with IL-12, activates established-Th1 cells to produce IFN-γ. Thus, IL-12 and IL-18 are considered pro-inflammatory cytokines that facilitate type-1 responses (67, 73). However, previous studies demonstrated that gene knockouts of IL-12, IL-18, and IFN-γ all exhibited similar lesion sizes as wild-type controls (65, 74). Recombinant IL-12-infused wild-type mice also showed similar bone resorption to controls. The findings with IFN-γ

were not confirmed in another study which reported that IFN-γ-deficient$^{(-/-)}$ mice presented with periapical lesions larger than those in wild-type animals (75). The expression level of IL-1 in periapical lesions was unchanged in these mice (74). Taken together, these results indicate that none of these cytokines has a non-redundant function in mediating periapical bone resorption.

IL-6, another macrophage-derived cytokine, was also detected in inflamed periapical tissue (76, 77). Its expression was found to be transiently increased on day 14 after infection and decreased in the chronic phase (43). IL-6 is a well-known pro-inflammatory cytokine, promoting bone resorption *via* osteoclastogenesis (78–80). Recent research has demonstrated that IL-6 also has anti-inflammatory effects by promoting macrophage IL-1RA secretion (81) and bone-forming effects by enhancing osteoblast differentiation (82–84). Previously, the protective role of IL-6 in periapical lesions was showed *in vivo*. Bone destruction was significantly increased in IL-6$^{-/-}$ mice versus in wild-type mice (69, 85). IL-6 antibody-mediated neutralization also increased bone resorption compared to untreated controls. In IL-6$^{-/-}$ mice, increased bone resorption importantly correlated with osteoclast count and IL-1 expression in periapical lesions, and inversely with anti-inflammatory IL-10 expression (69).

Both IL-4 and IL-10 are increased in periapical lesions (69). IL-4 is an anti-inflammatory cytokine playing pleiotropic roles in inflammation (86, 87). IL-10, a potent anti-inflammatory cytokine produced by regulatory T cells (Treg), macrophages, dendritic cells, Th 2 cells, and Th1 cells, among other immune cells (88–90). However, IL-4 and IL-10 have different anti-inflammatory effects on macrophages. In macrophages stimulated by oral pathogens, recombinant IL-10 inhibited IL-1α production, whereas recombinant IL-4 had no significant suppressive effect (91). Consistent with these *in vitro* findings, IL-10$^{-/-}$ mice exhibited significantly greater infection-stimulated bone resorption than wild-type mice, as well as markedly elevated IL-1 production in periapical inflammatory tissues (91). In contrast, there was no difference in periapical lesion size between IL-4$^{-/-}$ and wild-type mice (75, 91).

IL-17 is a pleiotropic cytokine produced by Th17 cells that induces a myriad of pro-inflammatory mediators (92). The expression of IL-17 was increased in infection-induced periapical lesions (65) and was significantly higher in symptomatic versus asymptomatic lesions (93). IL-17 induces human macrophages to produce and secrete pro-inflammatory cytokines IL-1β and TNF-α *in vitro* (94). IL-17A$^{-/-}$ mice were resistant to periapical lesions versus wild-type controls (65). However, IL-17 receptor type A-deficient (IL-17RA$^{-/-}$) mice conversely exhibited significantly increased bone destruction and inflammation. The expression of IL-1 was significantly upregulated in IL-17RA$^{-/-}$ lesions *in vivo* and IL-17RA$^{-/-}$ macrophages *in vitro*. The lesion size of IL-17RA$^{-/-}$ mice was decreased by IL-1β neutralization (95). IL-17A utilizes two IL-17 receptors, and IL-17RA has four ligands (96), therefore, this system must be meticulously dissected to comprehend these data. Nevertheless, IL-17RA signaling likely plays a protective role in periapical lesions *via* IL-1 signaling and neutrophil priming.

TABLE 1 The effect of deficiency/neutralization of each cytokine or receptor on periapical lesion.

| | Cytokine/ receptor | Effect on lesion size/ abscess | IL-1 level in lesion | References |
|---|---|---|---|---|
| Neutralization | IL-1β[a] | Down | | (95) |
| | IL-1α and IL-1β | Abscess formation | N.S. | (52) |
| Deficiency | IL-1R1 | Up | | (53, 54) |
| | TNF-α | N.S. | | (65) |
| | IL-17A | Drastically down | | (65) |
| | IL-17RA | Up | Up | (95) |
| | IFN-γ | N.S./up | N.S. | (65, 74, 75) |
| | IL-12 | N.S. | N.S. | (74) |
| | IL-18 | N.S. | N.S. | (74) |
| | IL-6 | Up | Up | (69, 85) |
| | IL-4 | N.S. | Down | (75, 91) |
| | IL-10 | Drastically up | Drastically up | (75, 91) |

[a]The effect of IL-1β neutralization was evaluated in IL-17RA$^{-/-}$ model.Blank, not evaluated; N.S, no significant.

Table 1 Summarizes the effect of cytokine or receptor deficiency/neutralization on periapical lesions. Although it is difficult to evaluate the effect of each cytokine because of their complex interactions (97), above reviewed experimental models suggest that anti-inflammatory cytokines such as IL-10 and, to a lesser extent, IL-6, are dominant and have non-redundant functions, compared to inflammatory cytokines in the immunomodulation of AP. In addition, the positive correlation between the IL-1 level and lesion size implies IL-1 is a principal cytokine in periapical lesion expansion and a useful biomarker for assessing inflammation.

## The impact of obesity and diabetes mellitus on periapical lesions

It is now widely accepted that obesity causes systemic low-grade chronic inflammation (98). As noted above, obesity increases the risk of severe inflammation (26), and predisposes to the development of postoperative and nosocomial infections, as well as serious complications of common infections (98, 99). Obesity also increases the risk for severe symptoms and poor prognosis in viral infections, including coronavirus disease 2019 (100). In the oral cavity, obesity correlates with the prevalence and severity of periodontitis (101). Deshpande et al. reported that obesity

worsens all gingival index, probing depth, gingival recession, and clinical attachment levels than non-obese patients (102).

Diabetes, as an obesity complication, also has negative effects on AP. Diabetes decreases the success rate of endodontic treatment in teeth with AP preoperatively, and increases the risk of post-treatment tooth loss (27, 28, 103–105). According to previous *in vivo* rodent studies, obesity significantly increases bone destruction in experimentally-induced AP (29–31). As discussed in the following section, several potential mechanisms underlying obesity-induced inflammation have been proposed, but the actual mechanism is not yet fully understood.

## Potential mechanism of obesity-exacerbating periapical bone destruction

Many studies provide evidence that obesity alters immune responses. In obesity, macrophages significantly accumulate in the white adipose tissue (106, 107); and the phenotype of accumulated macrophages possess a pro-inflammatory M1-polarized state, whereas resident macrophages in lean mice have a pro-resolving M2 phenotype (108–111). The M1-dominant adipose macrophages likely develop an inflammatory milieu (112). The circulating levels of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β was elevated in obese subjects (113, 114). Chronic exposure to these cytokines potentially causes insulin resistance resulting in hyperglycemia (115, 116). In addition, the serum levels of adipose tissue-derived cytokines, adipokines and adiponectin are also altered in the obese state. Obese adipose tissue increases inflammatory adipokines, including leptin, resistin, visfatin, IL-6, TNF-α, and monocyte chemoattractant protein-1, while decreasing anti-inflammatory adipokines, including adiponectin, omentin, IL-10, and IL-4. The dysregulation of adipokine production may alter cellular immune function and contribute to chronic low-grade inflammation and disease pathology (117–119). Obesity also increases the populations of activated CD4+ and CD8+ T cells in adipose tissue (120) and significantly reduces circulating $T_{reg}$ cells (121–123) which may sustain low-grade chronic inflammation. Furthermore, obesity induces thymic involution and convergent T cell repertoire, impairing impaired immune responses and increasing the risk and severity of infections (124).

As noted above, the effects of obesity on immune function are manifold. However, it remains unclear how obesity is associated with the expansion of periapical bone destruction. Therefore, our group examined possible pathways involved in bone loss in obesity using bulk-mRNA next-generation sequencing analysis. Comprehensive gene expression analysis revealed that, among a total 15,029 expressed genes, only 51 were differentially expressed in periapical lesions in DIO-B6

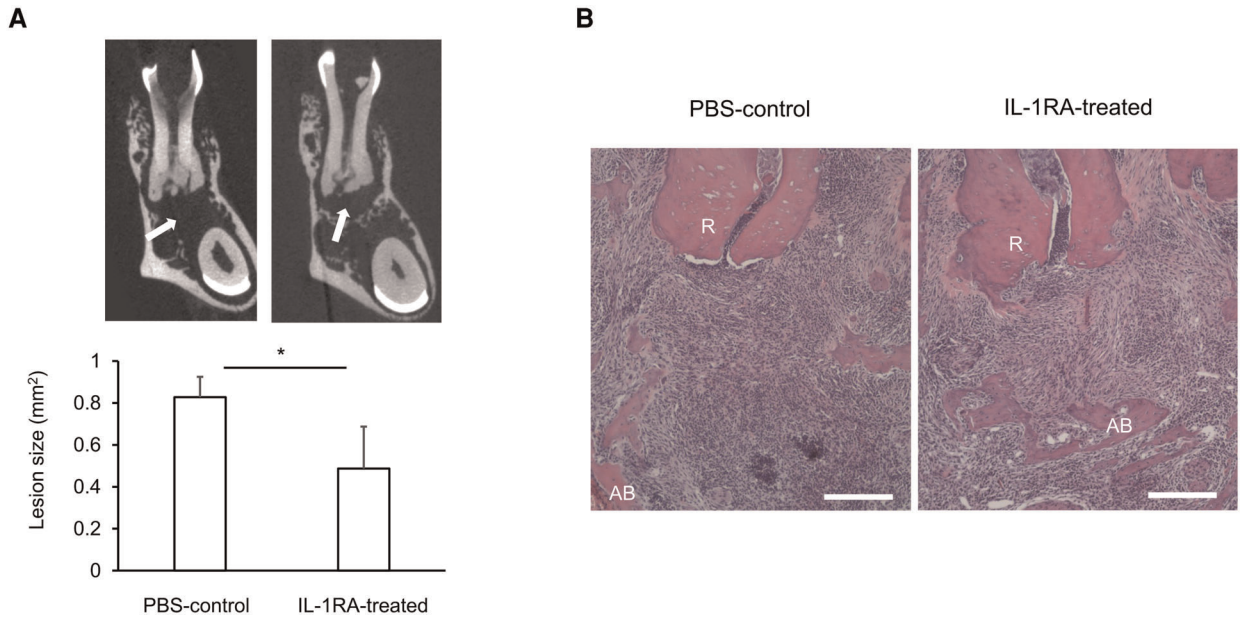



FIGURE 1
(**A**) Representative microCT images and periapical lesion size in phosphate buffered saline (PBS)-control and IL-1RA administration on day 42 after endodontic infection. Values are mean and SD; *$p < 0.05$. Arrow: periapical lesion. (**B**) Histology of periapical lesions on day 42 after endodontic infection. Representative images of hematoxylin and eosin (HE) staining for each group are shown. PBS, phosphate buffered saline; AB, alveolar bone; R, dental root; Scale bars = 200 μm.

mice versus lean controls. Among them, *Il1rn* encoding IL-1RA was remarkably down-regulated (Log2 fold change = −1.18, False Discovery Rate ($q$-value) = 0.0002). At the same time, *Il1a*, but not *Il1b*, was also decreased (−0.994-fold, $q = 0.046$) (31). These results suggest DIO impairs IL-1RA-dependent homeostatic suppression of IL-1 signaling, at least in the local environment.

Systemically, significantly increased IL-1 serum levels (114, 125) likely contribute to worsening of insulin resistance under obese conditions (116). However, given the lack of significant changes in the expression of IL-1 signaling genes, including NF-κB, in AP (31), systemically increased IL-1 may have little effect on AP. Interestingly, IL-1RA serum levels are also elevated in obesity (126). However, the concentration of IL-1RA is likely insufficient to block the effects of elevated IL-1. Indeed, administration of IL-1RA improves insulin sensitivity in animal models of obesity (116), suggesting IL-1RA-dependent homeostatic regulation of IL-1 signaling is not fully functional in obesity. We therefore examined if a decrease or loss of IL-1RA contributes to obesity-associated periapical inflammation by IL-1RA administration in infected DIO-B6 mice. Remarkably, periapical bone destruction was inhibited by 41.2% by IL-1RA (**Figure 1A**, $p < 0.05$). Histological analysis revealed that IL-1RA-treated mice showed less inflammatory cell infiltration and well-developed fibrosis (**Figure 1B**). These results indicate that inflammation was down-regulated by IL-1RA, and that the lesion was composed mainly of mature granulation tissue compared to the immune granulomas in controls. Therefore, immunomodulation by IL-1RA is likely important for the control of AP, even in obesity.

Appropriate regulation of IL-1 signaling according to the host and infection status may lead to an optimal immune/inflammatory response in terms of timely onset/resolution and adequate host defense. In the first section, we explained that excessive IL-1 and its signaling cause exacerbation of AP in the non-obese state. At the same time, IL-1RA homeostatically regulates IL-1 signaling, suppressing excessive IL-1-mediated responses. In the second section, we described that obesity dysregulates IL-1RA-dependent homeostatic IL-1 signaling regulation and causes chronic elevation of inflammation, tissue destruction, and prolonged healing. Endodontic infection in DIO may exacerbate bone destruction in the long term *via chronically* elevating IL-1 signaling at a low level due to downregulation of *Il1rn*. However, the role of IL-1 signaling is diverse and complex. The impact of IL-1 signaling on both systemic and local conditions has not been fully understood. Thus, further studies are essential for the changes in IL-1 signaling associated with various systemic conditions, the underlying mechanisms, and infection-stimulated bone destruction.

## Author contributions

PS and HS designed the study. KT and MMAP performed experiments in DIO mice. HF contributed to histological analysis. KT, MMAP, HF, and HS analyzed the data. KT, HS, and PS prepared the manuscript. All authors contributed to the article and approved the submitted version.

## Funding

This study was supported in part by a grant from National Institute of Dental and Craniofacial Research/National Institutes of Health (R01DE024796 to HS).

## Acknowledgments

We thank Dr. Mutsumi Miyauchi (Department of Oral and Maxillofacial pathobiology Graduate School of Biomedical and Health Sciences Hiroshima University) for co-operating histological analysis.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## References

1. Kakehashi S, Stanley HR, Fitzgerald RJ. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. *Oral Surg Oral Med Oral Pathol.* (1965) 20:340–9. doi: 10.1016/0030-4220(65)90166-0

2. Chen L, Deng H, Cui H, Fang J, Zuo Z, Deng J, et al. Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget.* (2017) 9:7204–18. doi: 10.18632/oncotarget.23208

3. Kawashima N, Okiji T, Kosaka T, Suda H. Kinetics of macrophages and lymphoid cells during the development of experimentally induced periapical lesions in rat molars: A quantitative immunohistochemical study. *J Endod*. (1996) 22:311–6. doi: 10.1016/S0099-2399(96)80266-4

4. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. *Front Immunol*. (2014) 5:461. doi: 10.3389/fimmu.2014.00461

5. Stashenko P, Wang CY, Riley E, Wu Y, Ostroff G, Niederman R. Reduction of infection-stimulated periapical bone resorption by the biological response modifier PGG glucan. *J Dent Res*. (1995) 74:323–30. doi: 10.1177/00220345950740010701

6. Kawashima N, Niederman R, Hynes RO, Ullmann-Cullere M, Stashenko P. Infection-stimulated infraosseus inflammation and bone destruction is increased in P-/E-selectin knockout mice. *Immunology*. (1999) 97:117–23. doi: 10.1046/j.1365-2567.1999.00754.x

7. Hansson GK, Libby P, Schönbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res*. (2002) 91:281–91. doi: 10.1161/01.res.0000029784.15893.10

8. Graunaite I, Lodiene G, Maciulskiene V. Pathogenesis of apical periodontitis: A literature review. *J Oral Maxillofac Res*. (2012) 2:e1. doi: 10.5037/jomr.2011.2401

9. Stashenko P, Teles R, D'Souza R. Periapical inflammatory responses and their modulation. *Crit Rev Oral Biol Med*. (1998) 9:498–521. doi: 10.1177/10454411980090040701

10. Stashenko P, Dewhirst FE, Peros WJ, Kent RL, Ago JM. Synergistic interactions between interleukin 1, tumor necrosis factor, and lymphotoxin in bone resorption. *J Immunol*. (1987) 138:1464–8.

11. Horton JE, Raisz LG, Simmons HA, Oppenheim JJ, Mergenhagen SE. Bone resorbing activity in supernatant fluid from cultured human peripheral blood leukocytes. *Science*. (1972) 177:793–5. doi: 10.1126/science.177.4051.793

12. Dewhirst FE, Stashenko PP, Mole JE, Tsurumachi T. Purification and partial sequence of human osteoclast-activating factor: Identity with interleukin 1 beta. *J Immunol*. (1985) 135:2562–8.

13. Wang CY, Stashenko P. Characterization of bone-resorbing activity in human periapical lesions. *J Endod*. (1993) 19:107–11. doi: 10.1016/S0099-2399(06)80503-0

14. Wang CY, Stashenko P. Kinetics of bone-resorbing activity in developing periapical lesions. *J Dent Res*. (1991) 70:1362–6. doi: 10.1177/00220345910700100901

15. Wang CY, Stashenko P. The role of interleukin-1 alpha in the pathogenesis of periapical bone destruction in a rat model system. *Oral Microbiol Immunol*. (1993) 8:50–6. doi: 10.1111/j.1399-302x.1993.tb00543.x

16. Popovska L, Dimova C, Evrosimovska B, Stojanovska V, Muratovska I, Cetenović B, et al. Relationship between IL-1β production and endodontic status of human periapical lesions. *Vojnosanitski Pregled*. (2017) 74:1134–9. doi: 10.2298/VSP151228309P

17. Miller GA, DeMayo T, Hutter JW. Production of interleukin-1 by polymorphonuclear leukocytes resident in periradicular tissue. *J Endod*. (1996) 22:346–51. doi: 10.1016/S0099-2399(96)80215-9

18. Gomes BPFA, Berber VB, Marinho ACS, Louzada LM, Arruda-Vasconcelos R, Passini MRZ, et al. Chemomechanical preparation influences the microbial community and the levels of LPS, LTA and cytokines in combined endodontic-periodontal lesions: A clinical study. *J Periodontal Res*. (2022) 57:341–56. doi: 10.1111/jre.12964

19. Maia LM, Espaladori MC, Diniz JMB, Tavares WLF, de Brito LCN, Vieira LQ, et al. Clinical endodontic procedures modulate periapical cytokine and chemokine gene expressions. *Clin Oral Investig*. (2020) 24:3691–7. doi: 10.1007/s00784-020-03247-y

20. Espaladori MC, Diniz JMB, de Brito LCN, Tavares WLF, Kawai T, Vieira LQ, et al. Selenium intracanal dressing: Effects on the periapical immune response. *Clin Oral Investig*. (2021) 25:2951–8. doi: 10.1007/s00784-020-03615-8

21. Martinho FC, Chiesa WM, Leite FR, Cirelli JA, Gomes BP. Correlation between clinical/radiographic features and inflammatory cytokine networks produced by macrophages stimulated with endodontic content. *J Endod*. (2012) 38:740–5. doi: 10.1016/j.joen.2012.02.021

22. Seckinger P, Klein-Nulend J, Alander C, Thompson RC, Dayer JM, Raisz LG. Natural and recombinant human IL-1 receptor antagonists block the effects of IL-1 on bone resorption and prostaglandin production. *J Immunol*. (1990) 145:4181–4.

23. Must A, Spadano J, Coakley EH, Field AE, Colditz G, Dietz WH. The disease burden associated with overweight and obesity. *JAMA*. (1999) 282:1523–9. doi: 10.1001/jama.282.16.1523

24. Centers for Disease Control and Prevention. The health effects of overweight and obesity (2021). Available at: https://www.cdc.gov/healthyweight/effects/index.html (Accessed May 05, 2021).

25. World Health Organization. Obesity and overweight (2021). Available at: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight (Accessed May 05, 2021).

26. Pugliese G, Liccardi A, Graziadio C, Barrea L, Muscogiuri G, Colao A. Obesity and infectious diseases: Pathophysiology and epidemiology of a double pandemic condition. *Int J Obes*. (2022) 46:449–65. doi: 10.1038/s41366-021-01035-6

27. Laukkanen E, Vehkalahti MM, Kotiranta AK. Impact of systemic diseases and tooth-based factors on outcome of root canal treatment. *Int Endod J*. (2019) 52:1417–26. doi: 10.1111/iej.13143

28. Segura-Egea JJ, Cabanillas-Balsera D, Jiménez-Sánchez MC, Martín-González J. Endodontics and diabetes: Association versus causation. *Int Endod J*. (2019) 52:790–802. doi: 10.1111/iej.13079

29. Tibúrcio-Machado CDS, Lang PM, Campos MM, Wolle CFB, Barcelos RCS, Zago NB, et al. High-fat diet effect on periapical lesions and hepatic enzymatic antioxidant in rats. *Life Sci*. (2021) 264:118637. doi: 10.1016/j.lfs.2020.118637

30. Brasil SC, Santos RMM, Fernandes A, Lima RS, Costa CAS, Pinto KMMDC, et al. Influence of a high-fat diet in the progression of apical periodontitis. *J Endod*. (2021) 47:600–5. doi: 10.1016/j.joen.2020.12.015

31. Azuma MM, Tazawa K, Furusho H, Sasaki H. Mechanisms of elevated periapical bone loss in diet-induced obesity. 2021 IADR/AADR/CADR General Session; 2021 July 21–24. *Virtual J Dent Res*. (2021) 100(Special Issue A):0380.

32. Sims JE, Smith DE. The IL-1 family: Regulators of immunity. *Nat Rev Immunol*. (2010) 10:89–102. doi: 10.1038/nri2691

33. Dinarello CA. The IL-1 family of cytokines and receptors in rheumatic diseases. *Nat Rev Rheumatol*. (2019) 15:612–32. doi: 10.1038/s41584-019-0277-8

34. Fields JK, Günther S, Sundberg EJ. Structural basis of IL-1 family cytokine signaling. *Front Immunol*. (2019) 10:1412. doi: 10.3389/fimmu.2019.01412

35. Dinarello CA. Interleukin-1 and interleukin-1 antagonism. *Blood*. (1991) 77:1627–52. doi: 10.1182/blood.V77.8.1627.1627

36. Stashenko P, Dewhirst FE, Rooney ML, Desjardins LA, Heeley JD. Interleukin-1 beta is a potent inhibitor of bone formation in vitro. *J Bone Miner Res*. (1987) 2:559–65. doi: 10.1002/jbmr.5650020612

37. Amarasekara DS, Yun H, Kim S, Lee N, Kim H, Rho J. Regulation of osteoclast differentiation by cytokine networks. *Immune Netw*. (2018) 18:e8. doi: 10.4110/in.2018.18.e8

38. Jimi E, Nakamura I, Duong LT, Ikebe T, Takahashi N, Rodan GA, et al. Interleukin 1 induces multinucleation and bone-resorbing activity of osteoclasts in the absence of osteoblasts/stromal cells. *Exp Cell Res*. (1999) 247:84–93. doi: 10.1006/excr.1998.4320

39. Nakamura I, Jimi E. Regulation of osteoclast differentiation and function by interleukin-1. *Vitam Horm*. (2006) 74:357–70. doi: 10.1016/S0083-6729(06)74015-8

40. Kim JH, Jin HM, Kim K, Song I, Youn BU, Matsuo K, et al. The mechanism of osteoclast differentiation induced by IL-1. *J Immunol*. (2009) 183:1862–70. doi: 10.4049/jimmunol.0803007

41. Jimi E, Nakamura I, Ikebe T, Akiyama S, Takahashi N, Suda T. Activation of NF-kappaB is involved in the survival of osteoclasts promoted by interleukin-1. *J Biol Chem*. (1998) 273:8799–805. doi: 10.1074/jbc.273.15.8799

42. Miyazaki T, Katagiri H, Kanegae Y, Takayanagi H, Sawada Y, Yamamoto A, et al. Reciprocal role of ERK and NF-kappaB pathways in survival and activation of osteoclasts. *J Cell Biol*. (2000) 148:333–42. doi: 10.1083/jcb.148.2.333

43. Kawashima N, Stashenko P. Expression of bone-resorptive and regulatory cytokines in murine periapical inflammation. *Arch Oral Biol*. (1999) 44:55–66. doi: 10.1016/s0003-9969(98)00094-6

44. Wang CY, Tani-Ishii N, Stashenko P. Bone-resorptive cytokine gene expression in periapical lesions in the rat. *Oral Microbiol Immunol*. (1997) 12:65–71. doi: 10.1111/j.1399-302x.1997.tb00619.x

45. Matsuo T, Ebisu S, Nakanishi T, Yonemura K, Harada Y, Okada H. Interleukin-1 alpha and interleukin-1 beta periapical exudates of infected root canals: Correlations with the clinical findings of the involved teeth. *J Endod*. (1994) 20:432–5. doi: 10.1016/s0099-2399(06)80032-4

46. Martinez ZR, Naruishi K, Yamashiro K, Myokai F, Yamada T, Matsuura K, et al. Gene profiles during root canal treatment in experimental rat periapical lesions. *J Endod*. (2007) 33:936–43. doi: 10.1016/j.joen.2007.04.016

47. Wang G, Sweren E, Liu H, Wier E, Alphonse MP, Chen R, et al. Bacteria induce skin regeneration via IL-1β signaling. *Cell Host Microbe*. (2021) 29:777–791.e6. doi: 10.1016/j.chom.2021.03.003

48. Mosley B, Urdal DL, Prickett KS, Larsen A, Cosman D, Conlon PJ, et al. The interleukin-1 receptor binds the human interleukin-1 alpha precursor but not the interleukin-1 beta precursor. *J Biol Chem*. (1987) 262:2941–4. doi: 10.1016/S0021-9258(18)61450-4

49. Tani-Ishii N, Wang CY, Stashenko P. Immunolocalization of bone-resorptive cytokines in rat pulp and periapical lesions following surgical pulp exposure. *Oral Microbiol Immunol*. (1995) 10:213–9. doi: 10.1111/j.1399-302x

50. Jakovljevic A, Knezevic A, Karalic D, Soldatovic I, Popovic B, Milasin J, et al. Pro-inflammatory cytokine levels in human apical periodontitis: Correlation with clinical and histological findings. *Aust Endod J*. (2015) 41:72–7. doi: 10.1111/aej.12072

51. Yang NY, Zhou Y, Zhao HY, Liu XY, Sun Z, Shang JJ. Increased interleukin 1$\alpha$ and interleukin 1$\beta$ expression is involved in the progression of periapical lesions in primary teeth. *BMC Oral Health*. (2018) 18:124. doi: 10.1186/s12903-018-0586-3

52. Youssef H, Stashenko P. Interleukin-1 and estrogen protect against disseminating dentoalveolar infections. *Int J Oral Sci*. (2017) 9:16–23. doi: 10.1038/ijos.2016.61

53. Chen CP, Hertzberg M, Jiang Y, Graves DT. Interleukin-1 and tumor necrosis factor receptor signaling is not required for bacteria-induced osteoclastogenesis and bone loss but is essential for protecting the host from a mixed anaerobic infection. *Am J Pathol*. (1999) 155:2145–52. doi: 10.1016/S0002-9440(10)65532-0

54. Graves DT, Chen CP, Douville C, Jiang Y. Interleukin-1 receptor signaling rather than that of tumor necrosis factor is critical in protecting the host from the severe consequences of a polymicrobe anaerobic infection. *Infect Immun*. (2000) 68:4746–51. doi: 10.1128/IAI.68.8.4746-4751.2000

55. Opal SM, DePalo VA. Anti-inflammatory cytokines. *Chest*. (2000) 117:1162–72. doi: 10.1378/chest.117.4.1162

56. Svenson M, Hansen MB, Heegaard P, Abell K, Bendtzen K. Specific binding of interleukin 1 (IL-1) beta and IL-1 receptor antagonist (IL-1ra) to human serum. High-affinity binding of IL-1ra to soluble IL-1 receptor type I. *Cytokine*. (1993) 5:427–35. doi: 10.1016/1043-4666(93)90032-z

57. Stashenko P, Wang CY, Tani-Ishii N, Yu SM. Pathogenesis of induced rat periapical lesions. *Oral Surg Oral Med Oral Pathol*. (1994) 78:494–502. doi: 10.1016/0030-4220(94)90044-2

58. Arend WP, Guthridge CJ. Biological role of interleukin 1 receptor antagonist isoforms. *Ann Rheum Dis*. (2000) 59(Suppl 1):i60–4. doi: 10.1136/ard.59.suppl_1.i60

59. Shimauchi H, Takayama S, Imai-Tanaka T, Okada H. Balance of interleukin-1 beta and interleukin-1 receptor antagonist in human periapical lesions. *J Endod*. (1998) 24:116–9. doi: 10.1016/s0099-2399(98)80089-7

60. Cavaillon JM. Cytokines and macrophages. *Biomed Pharmacother*. (1994) 48:445–53. doi: 10.1016/0753-3322(94)90005-1

61. Parameswaran N, Patial S. Tumor necrosis factor-α signaling in macrophages. *Crit Rev Eukaryot Gene Expr*. (2010) 20:87–103. doi: 10.1615/critreveukargeneexpr.v20.i2.10

62. Bachwich PR, Chensue SW, Larrick JW, Kunkel SL. Tumor necrosis factor stimulates interleukin-1 and prostaglandin E2 production in resting macrophages. *Biochem Biophys Res Commun*. (1986) 136:94–101. doi: 10.1016/0006-291x(86)90881-8

63. Luo G, Li F, Li X, Wang ZG, Zhang B. TNF-*A* and RANKL promote osteoclastogenesis by upregulating RANK via the NF-κB pathway. *Mol Med Rep*. (2018) 17:6605–11. doi: 10.3892/mmr.2018.8698

64. Marahleh A, Kitaura H, Ohori F, Kishikawa A, Ogawa S, Shen WR, et al. TNF-α Directly enhances osteocyte RANKL expression and promotes osteoclast formation. *Front Immunol*. (2019) 10:2925. doi: 10.3389/fimmu.2019.02925

65. Oseko F, Yamamoto T, Akamatsu Y, Kanamura N, Iwakura Y, Imanishi J, et al. IL-17 is involved in bone resorption in mouse periapical lesions. *Microbiol Immunol*. (2009) 53:287–94. doi: 10.1111/j.1348-0421.2009.00123.x

66. Kopitar-Jerala N. The role of interferons in inflammation and inflammasome activation. *Front Immunol*. (2017) 8:873. doi: 10.3389/fimmu.2017.00873

67. Nakanishi K. Unique action of interleukin-18 on T cells and other immune cells. *Front Immunol*. (2018) 9:763. doi: 10.3389/fimmu.2018.00763

68. Bohn E, Autenrieth IB. IL-12 is essential for resistance against Yersinia enterocolitica by triggering IFN-gamma production in NK cells and CD4+ T cells. *J Immunol*. (1996) 156:1458–68.

69. Balto K, Sasaki H, Stashenko P. Interleukin-6 deficiency increases inflammatory bone destruction. *Infect Immun*. (2001) 69:744–50. doi: 10.1128/IAI.69.2.744-750.2001

70. Liu S, Li Q, Liu Y. Immunohistochemical localization of NALP3 inflammasome in experimental periapical lesions. *Int Endod J*. (2014) 47:949–57. doi: 10.1111/iej.12240

71. Haq AU, Rinehart JJ, Maca RD. The effect of gamma interferon on IL-1 secretion of in vitro differentiated human macrophages. *J Leukoc Biol*. (1985) 38:735–46. doi: 10.1002/jlb.38.6.735

72. Chujor CS, Klein L, Lam C. Selective inhibition of interleukin-1 beta gene expression in activated RAW 264.7 macrophages by interferon-gamma. *Eur J Immunol*. (1996) 26:1253–9. doi: 10.1002/eji.1830260611

73. Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science*. (1993) 260:547–9. doi: 10.1126/science.8097338

74. Sasaki H, Balto K, Kawashima N, Eastcott J, Hoshino K, Akira S, et al. Gamma interferon (IFN-gamma) and IFN-gamma-inducing cytokines interleukin-12 (IL-12) and IL-18 do not augment infection-stimulated bone resorption in vivo. *Clin Diagn Lab Immunol*. (2004) 11:106–10. doi: 10.1128/cdli.11.1.106-110.2004

75. De Rossi A, Rocha LB, Rossi MA. Interferon-gamma, interleukin-10, intercellular adhesion molecule-1, and chemokine receptor 5, but not interleukin-4, attenuate the development of periapical lesions. *J Endod*. (2008) 34:31–8. doi: 10.1016/j.joen.2007.09.021

76. Prso IB, Kocjan W, Simić H, Brumini G, Pezelj-Ribarić S, Borcić J, et al. Tumor necrosis factor-alpha and interleukin 6 in human periapical lesions. *Mediators Inflamm*. (2007):38210. doi: 10.1155/2007/38210

77. Bando Y, Henderson B, Meghji S, Poole S, Harris M. Immunocytochemical localization of inflammatory cytokines and vascular adhesion receptors in radicular cysts. *J Oral Pathol Med*. (1993) 22:221–7. doi: 10.1111/j.1600-0714.1993.tb01060.x

78. Ishimi Y, Miyaura C, Jin CH, Akatsu T, Abe E, Nakamura Y, et al. IL-6 is produced by osteoblasts and induces bone resorption. *J Immunol*. (1990) 145:3297–303.

79. Tat S K, Padrines M, Théoleyre S, Heymann D, Fortun Y. IL-6, RANKL, TNF-alpha/IL-1: Interrelations in bone resorption pathophysiology. *Cytokine Growth Factor Rev*. (2004) 15:49–60. doi: 10.1016/j.cytogfr.2003.10.005

80. Hughes FJ, Howells GL. Interleukin-6 inhibits bone formation in vitro. *Bone Miner*. (1993) 21:21–8. doi: 10.1016/s0169-6009(08)80117-1

81. Tilg H, Trehu E, Atkins MB, Dinarello CA, Mier JW. Interleukin-6 (IL-6) as an anti-inflammatory cytokine: Induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood*. (1994) 83:113–8. doi: 10.1182/blood.V83.1.113.113

82. Blanchard F, Duplomb L, Baud'huin M, Brounais B. The dual role of IL-6-type cytokines on bone remodeling and bone tumors. *Cytokine Growth Factor Rev*. (2009) 20:19–28. doi: 10.1016/j.cytogfr.2008.11.004

83. Itoh S, Udagawa N, Takahashi N, Yoshitake F, Narita H, Ebisu S, et al. A critical role for interleukin-6 family-mediated Stat3 activation in osteoblast differentiation and bone formation. *Bone*. (2006) 39:505–12. doi: 10.1016/j.bone.2006.02.074

84. Sims NA. Cell-specific paracrine actions of IL-6 family cytokines from bone, marrow and muscle that control bone formation and resorption. *Int J Biochem Cell Biol*. (2016) 79:14–23. doi: 10.1016/j.biocel.2016.08.003

85. Huang GT, Do M, Wingard M, Park JS, Chugal N. Effect of interleukin-6 deficiency on the formation of periapical lesions after pulp exposure in mice. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. (2001) 92:83–8. doi: 10.1067/moe.2001.115025

86. Chatterjee P, Chiasson VL, Bounds KR, Mitchell BM. Regulation of the anti-inflammatory cytokines interleukin-4 and interleukin-10 during pregnancy. *Front Immunol*. (2014) 5:253. doi: 10.3389/fimmu.2014.00253

87. Gadani SP, Cronk JC, Norris GT, Kipnis J. IL-4 in the brain: A cytokine to remember. *J Immunol*. (2012) 189:4213–9. doi: 10.4049/jimmunol.1202246

88. Sabat R, Grütz G, Warszawska K, Kirsch S, Witte E, Wolk K, et al. Biology of interleukin-10. *Cytokine Growth Factor Rev*. (2010) 21:331–44. doi: 10.1016/j.cytogfr.2010.09.002

89. Trinchieri G. Interleukin-10 production by effector T cells: Th1 cells show self control. *J Exp Med*. (2007) 204:239–43. doi: 10.1084/jem.20070104

90. Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J Immunol*. (1993) 150:353–60.

91. Sasaki H, Hou L, Belani A, Wang CY, Uchiyama T, Müller R, et al. IL-10, but not IL-4, suppresses infection-stimulated bone resorption in vivo. *J Immunol*. (2000) 165:3626–30. doi: 10.4049/jimmunol.165.7.3626

92. Cua DJ, Tato CM. Innate IL-17-producing cells: The sentinels of the immune system. *Nat Rev Immunol*. (2010) 10:479–89. doi: 10.1038/nri2800

93. Colić M, Vasilijić S, Gazivoda D, Vucević D, Marjanović M, Lukić A. Interleukin-17 plays a role in exacerbation of inflammation within chronic periapical lesions. *Eur J Oral Sci*. (2007) 115:315–20. doi: 10.1111/j.1600-0722.2007.00460.x

94. Jovanovic DV, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M, et al. IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol*. (1998) 160(7):3513–21

95. AlShwaimi E, Berggreen E, Furusho H, Rossall JC, Dobeck J, Yoganathan S, et al. IL-17 receptor A signaling is protective in infection-stimulated periapical bone destruction. *J Immunol*. (2013) 191:1785–91. doi: 10.4049/jimmunol.1202194

96. Gaffen SL. Structure and signalling in the IL-17 receptor family. *Nat Rev Immunol*. (2009) 9:556–67. doi: 10.1038/nri2586

97. Elenkov IJ, Iezzoni DG, Daly A, Harris AG, Chrousos GP. Cytokine dysregulation, inflammation and well-being. *Neuroimmunomodulation*. (2005) 12:255–69. doi: 10.1159/000087104

98. De Heredia FP, Gómez-Martínez S, Marcos A. Obesity, inflammation and the immune system. *Proc Nutr Soc*. (2012) 71:332–8. doi: 10.1017/S0029665112000092

99. Falagas ME, Kompoti M. Obesity and infection. *Lancet Infect Dis*. (2006) 6:438–46. doi: 10.1016/S1473-3099(06)70523-0

100. Petrakis D, Margină D, Tsarouhas K, Tekos F, Stan M, Nikitovic D, et al. Obesity - a risk factor for increased COVID-19 prevalence, severity and lethality (review). *Mol Med Rep*. (2020) 22:9–19. doi: 10.3892/mmr.2020.11127

101. Martinez-Herrera M, Silvestre-Rangil J, Silvestre FJ. Association between obesity and periodontal disease. A systematic review of epidemiological studies and controlled clinical trials. *Med Oral Patol Oral Cir Bucal*. (2017) 22:e708–15. doi: 10.4317/medoral.21786

102. Deshpande NC, Amrutiya MR. Obesity and oral health - is there a link? An observational study. *J Indian Soc Periodontol*. (2017) 21:229–33. doi: 10.4103/jisp.jisp_305_16

103. Segura-Egea JJ, Martín-González J, Cabanillas-Balsera D, Fouad AF, Velasco-Ortega E, López-López J. Association between diabetes and the prevalence of radiolucent periapical lesions in root-filled teeth: Systematic review and meta-analysis. *Clin Oral Investig*. (2016) 20:1133–41. doi: 10.1007/s00784-016-1805-4

104. Gupta A, Aggarwal V, Mehta N, Abraham D, Singh A. Diabetes mellitus and the healing of periapical lesions in root filled teeth: A systematic review and meta-analysis. *Int Endod J*. (2020) 53:1472–84. doi: 10.1111/iej.13366

105. Fouad AF, Burleson J. The effect of diabetes mellitus on endodontic treatment outcome: Data from an electronic patient record. *J Am Dent Assoc*. (2003) 134:43–51; quiz 117–8. doi: 10.14219/jada.archive.2003.0016.

106. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante Jr AW. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest*. (2003) 112:1796–808. doi: 10.1172/JCI19246

107. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest*. (2003) 112:1821–30. doi: 10.1172/JCI19451

108. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest*. (2007) 117:175–84. doi: 10.1172/JCI29881

109. Li P, Lu M, Nguyen MTA, Bae EJ, Chapman J, Feng D, et al. Functional heterogeneity of CD11c-positive adipose tissue macrophages in diet-induced obese mice. *J Biol Chem*. (2010) 285(20):15333–45. doi: 10.1074/jbc.M110.100263

110. Prieur X, Mok CY, Velagapudi VR, Núñez V, Fuentes L, Montaner D, et al. Differential lipid partitioning between adipocytes and tissue macrophages modulates macrophage lipotoxicity and M2/M1 polarization in obese mice. *Diabetes*. (2011) 60:797–809. doi: 10.2337/db10-0705

111. Lumeng CN, DelProposto JB, Westcott DJ, Saltiel AR. Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. *Diabetes*. (2008) 57:3239–46. doi: 10.2337/db08-0872

112. Morris DL, Singer K, Lumeng CN. Adipose tissue macrophages: Phenotypic plasticity and diversity in lean and obese states. *Curr Opin Clin Nutr Metab Care*. (2011) 14:341–6. doi: 10.1097/MCO.0b013e328347970b

113. Park HS, Park JY, Yu R. Relationship of obesity and visceral adiposity with serum concentrations of CRP, TNF-alpha and IL-6. *Diabetes Res Clin Pract*. (2005) 69:29–35. doi: 10.1016/j.diabres.2004.11.007

114. Palomera LF, Gómez-Arauz AY, Villanueva-Ortega E, Meléndez-Mier G, Islas-Andrade SA, Escobedo G. Serum levels of interleukin-1 beta associate better with severity of simple steatosis than liver function tests in morbidly obese patients. *J Res Med Sci*. (2018) 23:93. doi: 10.4103/jrms.JRMS_142_18

115. Rodríguez-Hernández H, Simental-Mendía LE, Rodríguez-Ramírez G, Reyes-Romero MA. Obesity and inflammation: Epidemiology, risk factors, and markers of inflammation. *Int J Endocrinol*. (2013):678159. doi: 10.1155/2013/678159

116. Ballak DB, Stienstra R, Tack CJ, Dinarello CA, van Diepen JA. IL-1 family members in the pathogenesis and treatment of metabolic disease: Focus on adipose tissue inflammation and insulin resistance. *Cytokine*. (2015) 75:280–90. doi: 10.1016/j.cyto.2015.05.005

117. Tilg H, Moschen AR. Adipocytokines: Mediators linking adipose tissue, inflammation and immunity. *Nat Rev Immunol*. (2006) 6:772–83. doi: 10.1038/nri1937

118. Taylor EB. The complex role of adipokines in obesity, inflammation, and autoimmunity. *Clin Sci*. (2021) 135:731–52. doi: 10.1042/CS20200895

119. Carbone F, La Rocca C, Matarese G. Immunological functions of leptin and adiponectin. *Biochimie*. (2012) 94:2082–8. doi: 10.1016/j.biochi.2012.05.018

120. Kiran S, Kumar V, Murphy EA, Enos RT, Singh UP. High fat diet-induced CD8+ T cells in adipose tissue mediate macrophages to sustain low-grade chronic inflammation. *Front Immunol*. (2021) 12:680944. doi: 10.3389/fimmu.2021.680944

121. Wagner NM, Brandhorst G, Czepluch F, Lankeit M, Eberle C, Herzberg S, et al. Circulating regulatory T cells are reduced in obesity and may identify subjects at increased metabolic and cardiovascular risk. *Obesity*. (2013) 21:461–8. doi: 10.1002/oby.20087

122. Agabiti-Rosei C, Trapletti V, Piantoni S, Airò P, Tincani A, De Ciuceis C, et al. Decreased circulating T regulatory lymphocytes in obese patients undergoing bariatric surgery. *PLoS One*. (2018) 13:e0197178. doi: 10.1371/journal.pone.0197178

123. Yun JM, Jialal I, Devaraj S. Effects of epigallocatechin gallate on regulatory T cell number and function in obese v. Lean volunteers. *Br J Nutr*. (2010) 103:1771–7. doi: 10.1017/S000711451000005X

124. Yang H, Youm YH, Vandanmagsar B, Rood J, Kumar KG, Butler AA, et al. Obesity accelerates thymic aging. *Blood*. (2009) 114:3803–12. doi: 10.1182/blood-2009-03-213595

125. Um JY, Rim HK, Kim SJ, Kim HL, Hong SH. Functional polymorphism of IL-1 alpha and its potential role in obesity in humans and mice. *PLoS One*. (2011) 6:e29524. doi: 10.1371/journal.pone.0029524

126. Meier CA, Bobbioni E, Gabay C, Assimacopoulos-Jeannet F, Golay A, Dayer JM. IL-1 receptor antagonist serum levels are increased in human obesity: A possible link to the resistance to leptin? *J Clin Endocrinol Metab*. (2002) 87:1184–8. doi: 10.1210/jcem.87.3.8351

frontiers | Frontiers in Oral Health

TYPE Original Research
PUBLISHED 18 August 2022
DOI 10.3389/froh.2022.956072



OPEN ACCESS

EDITED BY
Despina Koletsi,
University of Zurich, Switzerland

REVIEWED BY
Nathalia Carolina Fernandes Fagundes,
University of Alberta, Canada
Kevin Sheng-Kai Ma,
University of Pennsylvania,
United States

*CORRESPONDENCE
Pia Heikkilä
pia.heikkila@helsinki.fi

SPECIALTY SECTION
This article was submitted to Oral Epidemiology, a section of the journal Frontiers in Oral Health

RECEIVED 29 May 2022
ACCEPTED 18 July 2022
PUBLISHED 18 August 2022

CITATION
Heikkilä P, Niskanen L, But A, Sorsa T and Haukka J (2022) Oral health associated with incident diabetes but not other chronic diseases: A register-based cohort study. *Front. Oral. Health* 3:956072. doi: 10.3389/froh.2022.956072



# Oral health associated with incident diabetes but not other chronic diseases: A register-based cohort study

Pia Heikkilä[1]*, Leo Niskanen[2], Anna But[3], Timo Sorsa[1,4] and Jari Haukka[3,5]

[1]Department of Oral and Maxillofacial Diseases, Faculty of Medicine, University of Helsinki and Helsinki University Hospital, Helsinki, Finland, [2]Internal Medicine, Päijät-Häme Central Hospital Hospital and Universities of Helsinki and Eastern Finland, Lahti, Finland, [3]Department of Public Health, Clinicum, University of Helsinki, Helsinki, Finland, [4]Division of Periodontology, Department of Dental Medicine, Karolinska Institutet, Huddinge, Sweden, [5]Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland

**Introduction:** Oral infectious diseases are common chronic oral diseases characterized by a chronic inflammatory condition. We investigated chronic oral diseases as potential risk factors for systemic chronic diseases, diabetes mellitus, connective tissue diseases, seropositive rheumatoid arthritis, ulcerative colitis, and Crohn's disease, as well as severe psychotic and other severe mental disorders.

**Methods:** The cohort comprised 68,273 patients aged ≥ 29 years with at least one dental visit to the Helsinki City Health Services between 2001 and 2002. The cohort was linked to the data on death (Statistics Finland), cancer (Finnish Cancer Registry), and drug reimbursement (Finnish Social Insurance Institution) and followed until death or the end of 2013. The outcomes of interest were the incidences of chronic diseases measured starting with special refund medication, which means Social Insurance Institution partly or fully reimburses medication costs. Outcomes of interest were diabetes mellitus, connective tissue diseases, seropositive rheumatoid arthritis, ulcerative colitis and Crohn's disease, and severe mental disorders.

**Results:** The mean follow-up time was 9.8 years. About 25% of the study population had periodontitis, 17% caries, over 70% apical periodontitis, and 9% <24 teeth at the start of follow-up. Diabetes was the only chronic systemic condition associated with oral health variables. Having 24 to 27 teeth was associated with a higher incidence rate ratio (IRR) (1.21, 95% confidence interval 1.09–1.33) compared to having 28 or more teeth; the IRR for having 23 or less was 1.40 (1.22–1.60). Having periodontitis (1.10, 1.01–1.20), caries (1.12, 1.01–1.23), or apical periodontitis (1.16, 1.04–1.30) is also associated with a higher risk of diabetes.

**Conclusion:** Our epidemiological 10 years follow-up study suggests that the association exists between chronic oral diseases and diabetes, warranting close collaboration among patient's healthcare professionals.

KEYWORDS

**periodontitis, diabetes, oral infections, chronic diseases, chronic systemic diseases**

10.3389/froh.2022.956072

# Introduction

The two major oral infectious diseases, dental caries and periodontitis, are common chronic oral infectious diseases characterized by a chronic inflammatory condition, and their progressions are influenced by multiple factors [1]. Severe periodontitis affects 10% to 15% of the global adult population [2]. Periodontitis is associated with an increased risk for several chronic systemic diseases such as diabetes [2], inflammatory bowel disease [3], cancer [4], and cardiovascular diseases through systemic often low-grade inflammation as the etiopathogenic link [5–7]. Caries can lead to the formation of apical periodontitis (AP), also capable of promoting and affecting the course of various systemic diseases. The prevalence of AP in Europe has been reported to affect 61% of individuals and 14% of teeth and increase with age [8]. There may be a moderate risk and correlation between some systemic chronic diseases and endodontic pathologies. AP has also been related to cardiovascular diseases and diabetes [9].

Low-grade systemic and tissue inflammations precede diabetes onset and are often linked to insulin resistance and the development of diabetes and its complications [10, 11]. Since effective therapy and management of the periodontal disease are well-established, it is important to know for future prevention and control of diabetes whether periodontitis indeed plays a role in the development of diabetes and its potentially fatal complications [12]. However, evidence from clinical trials and observational studies is still scarce, and more follow-up studies are required [13]. A review of the effect of periodontal disease on diabetes with four studies, in total 22,230 individuals, reported significant adverse effects of periodontal disease on glycaemic control, diabetes complications, and development of type 2 (and possibly gestational) diabetes [2]. Because the evidence was scarce and partly not generalizable, we called for large-scale studies with long follow-ups.

In a recent review of inflammatory bowel disease (IBD) and oral health, we found a higher risk of periodontal disease and worse oral health in IBD patients than in non-IBD ones. This meta-analysis included only case-control studies. We stated that longitudinal studies are needed to establish a link between IBD and periodontal disease [3].

Abbreviations: AGE, advanced glycation end-products; AP, periodontitis; CPI, periodontal treatment need index; DMTF decayed/missing/filled teeth; DT, decayed teeth; FCR, Finnish Cancer Registry; IBD, inflammatory bowel disease; ICD-10, International Classification of Diseases; I, primary caries; IRR, incidence rate ratios; MMP, matrix metalloproteinases; NNH, number needed to harm; SII, Finnish Social Insurance Institution; SES, socioeconomic status.

Evidence from systematic reviews supports the association between PD and a higher risk of rheumatoid arthritis [14–16]. However, most epidemiologic outcomes derive from case-control studies with relatively small sample sizes. Additionally, some evidence from animal models suggests the connection between PD and rheumatoid arthritis [17, 18].

We hypothesized that oral health abnormalities could precede and/or promote tissue inflammation related to chronic systemic conditions. We, therefore, investigated the associations between oral health and the incidence of the following systemic conditions: diabetes, IBD, connective tissue disease, and psychosis. The rationale for choosing these diseases were that they are relatively common in the general population, and thus in the registered studies, the diagnosis is reliable and readily assessed. These disorders are also distinct clinical entities, but as a matter of fact, all these disorders may have some common origins, for example, tissue inflammation seems to characterize these all, even mental disorders. Further, patients with mental disorders are usually also socially disadvantaged and have poorer somatic health in general, therefore the inclusion of this diagnostic category serves also as an internal control of the findings making the results more robust in supporting the close relationship between glucose metabolism and periodontitis.

The setting is a population-based follow-up observational register study where the initiation of drug medication measures incidence for specific studied chronic conditions with documented reimbursement.

# Methods

## Study population

We used the data from the patient register of the Public Dental Service of the City of Helsinki to identify all individuals aged 29 years or more with at least one primary dental healthcare visit between 1 January 2001 and 31 December 2002. For these patients, follow-up data on deaths and causes of death were obtained from the register of deaths of Statistics Finland [19] through a computerized register linkage using the unique personal identification codes assigned to every resident in Finland. Along with the date of death, mortality data also included the cause of death coded according to the 10th revision of the International Classification of Diseases (ICD-10). In addition, data on socioeconomic status and education were obtained from Statistics Finland. The dental care data were also linked to the Drug Reimbursement Register of the Finnish Social Insurance Institution (SII). These drug prescription records, except for institutionalized patients, cover the entire study population. In Finland, patients with chronic or severe diseases, such as diabetes, are granted special reimbursement rights for outpatient medical treatment based on a physician's statement on their condition and need for medication [20]. The cancer

diagnosis data, date of diagnosis, and ICD-O-3 code [21] were obtained from the Finnish Cancer Registry (FCR). The FCR database contains data on virtually all cancers diagnosed in Finland since 1953. The coverage and accuracy of the Finnish Cancer Registry data are excellent [22, 23].

Altogether 71,200 patients visited the Public Dental Service of Helsinki from 2001 to 2002. We restricted the study population to those who had no history of cancer at the first visit, who were alive 2 years after the first visit, and were with data on the number of teeth and other information on dental status. The dentist has done full mouth examinations, including cariological status and periodontal status (probing, bleeding on probing) and panoramic radiographs/intraoral radiographs. Malignant diseases can promote and modify the development and courses of systemic inflammatory diseases [24–27]. The size of the final study population was 48,609 individuals. The follow-up started 2 years after the first visit and continued until the occurrence of the outcome, 31 December 2013, or death, whichever occurred first.

## Outcomes

The outcomes of interest were the incidence of several chronic diseases measured by starting a special refund right for medication. Special reimbursements of drug expenses are given to patients who have a statement from their doctor attesting to their condition and need for medication [20]. We used both SII refund groups and specific ICD10 codes included in SII groups (Supplementary Table 1) as outcomes. The following diseases were included: diabetes mellitus (SII code 103), connective tissue diseases (202), seropositive rheumatoid arthritis (M05), ulcerative colitis and Crohn's disease (208), Crohn's disease (K50), ulcerative colitis (K51), and severe psychotic and other severe mental disorders (112). Severe mental disorders were included as negative outcome control [28]. Any individuals with prevalent refund rights were excluded when incidence was studied.

## The measure of exposure and potential confounders

We utilized data from dental visits in the follow-up period starting 2 years after the first visit. Dentists use the classification of the Finnish SSI to record treatment measures provided, and these codes were used here. These data include procedure codes of dental treatment (gingivitis, periodontitis, caries, endodontic, surgery, and prosthesis), and information on dental status presented by a number of teeth and oral health indices, such as primary caries (I), number of decayed teeth (DT), decayed/missing/filled teeth (DMFT), and need for periodontal treatment due to periodontal pockets (CPI = the Community Periodontal Index). Exposure to periodontitis was defined as a binary variable (no/yes) based on periodontitis treatment procedure codes [24, 25].

Among potential confounders in this study were socio-demographic characteristics, such as age, sex, statins at baseline, and socioeconomic status, which were available for the entire study population. Statistics Finland's professions were categorized into eight broader categories, including unknown, to represent socioeconomic status (SES). Statin (ATC code C10A) use in baseline was determined from prescription data of SII. To account for dental status other than periodontitis, we used number of teeth (0–23, 24–27, 28–32), indices I (0, 1–2, 3–4, ≥5), DT (0, 1–2, 3–4, ≥5), DMFT (0–13, 14–18, 19–23, ≥24 according to quartiles), and CPI (0–1, 2, 3–4), number of healthy sextants (0, 1, 2–4, 5–6), number of toothless sextants (0, 1–6), and indicators of different dental treatments (yes/no) [29, 30]. I, DT, DMFT, and CPI indices were defined by taking the maximum value of those recorded during the dental visits within 2 years after the first visit. The number of healthy sextants was specified according to the first visit, and the number of toothless sextants was selected by the minimum value. For part of the study population, however, health indices were not available because it is not routinely recorded at every visit; these appointments were defined as follow-up visits. We excluded these individuals from the study population.

## Statistical method

Incidence was described with incidence rates and modeled with the Poisson regression model, and results were reported as incidence rate ratios (IRR). The following explanatory variables were included: sex, age, socioeconomic status, usage of statins in baseline (no, yes), number of teeth, I index, D index, DMF index, CPI, periodontitis (no, yes), caries (no, yes), and endodontic caries (no, yes). All calculations were carried out using the R language [31].

## Patient involvement and ethical considerations

No patients were involved in setting the research question or the outcome measures, nor were they involved in the study's recruitment, design, or implementation. Patients were not asked to interpret or disseminate results. The Ethical Committee of the Faculty of Medicine, University of Helsinki, Finland (01/2014), reviewed the protocol. Data permits were received from the Social Insurance Institute (SII) (68/522/2014), the National Institute for Health and Welfare (THL/1295/5.05.05/2014), and Statistics Finland (TK-53-1290-14). According to Finnish law, this is a register-based study with anonymous data and no patient contact; thus no consent from anonymized patients were required.

10.3389/froh.2022.956072

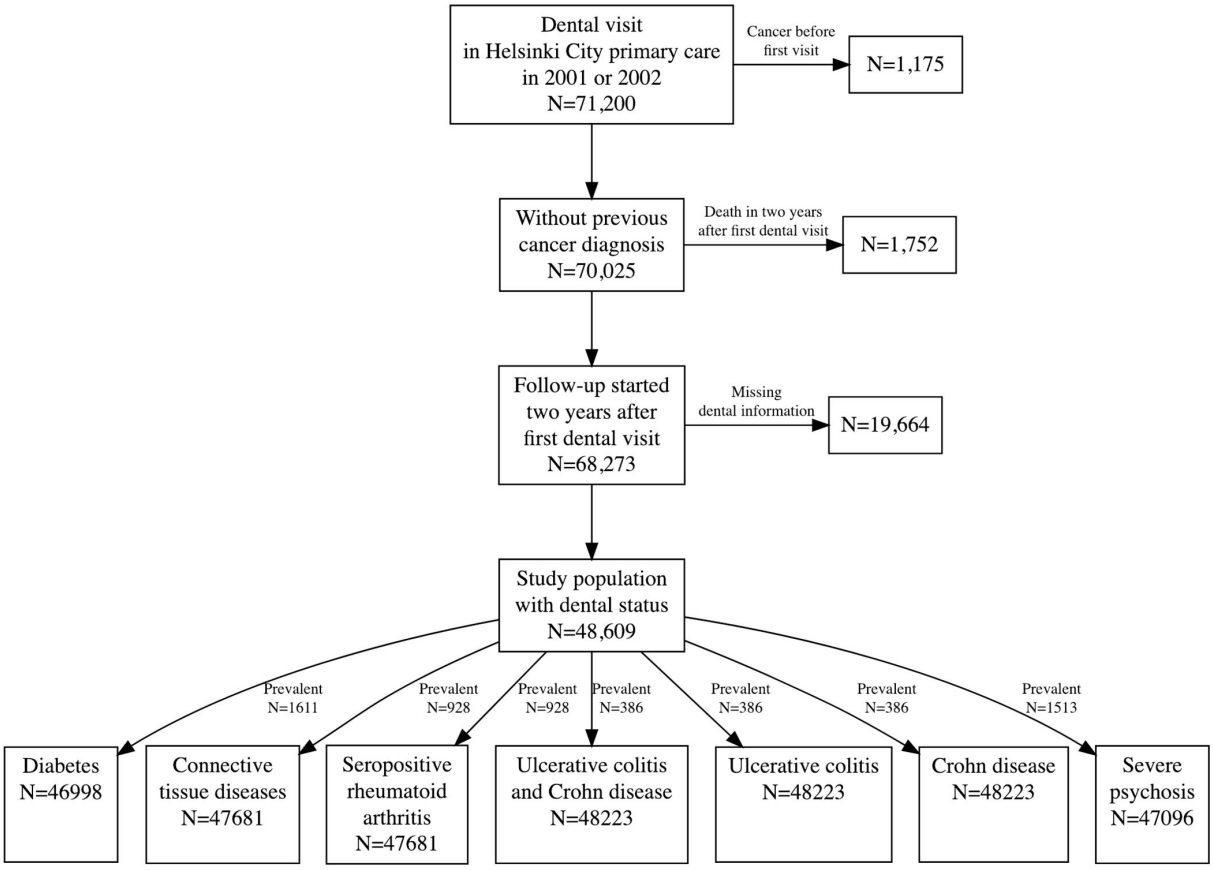



FIGURE 1
Flow chart of the study population construction.

## Results

The size of study cohorts varied between 46,998 for diabetes and 48,223 for IBD (ulcerative colitis and Crohn's disease; Figure 1 and Table 1). The mean follow-up time of diabetes mellitus was 9.7 years, connective tissue diseases 9.8, seropositive rheumatoid arthritis 9.9, ulcerative colitis and Crohn's disease 9.8, Crohn's disease 9.9, ulcerative colitis 9.8, and for severe psychotic and other severe mental disorders 9.8 years. The reason for different population sizes is that the prevalence of chronic conditions varies, being the highest for diabetes. About 25% of the study population had periodontitis, 17% caries, over 70% apical periodontitis, and 9% <24 teeth at the start of follow-up. The socioeconomic status of the study population and the general population of the City of Helsinki were quite similar (Supplementary Table 2). Diabetes was the only chronic condition associated with oral health variables (Table 2 and Figure 2). It turned out that having 24 to 27 teeth was associated with a higher incidence rate ratio (IRR) (1.21, 95% confidence interval 1.09–1.33) compared to having 28 or more teeth; IRR for having 23 or less was 1.40 (1.22–1.60). Having periodontitis (1.10, 1.01–1.20), caries (1.12, 1.01–1.23), or apical periodontitis lesions (1.16, 1.014–1.30) were also associated with a higher risk of diabetes (Table 2 and Supplementary Table 2). We observed a higher association between statin use and incidence of diabetes (2.49, 2.10- 2.94). There was also a relatively strong association between the number of decayed teeth (DT) and the incidence of diabetes. The IRR for DT 3–4 was 1.25 (1.10–1.42) compared to zero (Supplementary Table 3). We did not detect any other associations between oral health variables and the incidence of other chronic diseases.

We calculated the number needed to harm (NNH) for new-onset diabetes in 1 year for three oral health variables (Table 3). NNH for PD was 1,736 (95% confidence interval 903–22,103), for caries 1,342 (709, 12,497), and NNH for having <24 teeth compared to having 28 or more teeth was 262 (190–420).

## Discussion

We collected these from the largely presentative population (N = 68,273) observational register study with a long follow-up (10 years). The incidence is measured by the initiation of drug treatment for specific conditions verified by special

10.3389/froh.2022.956072

TABLE 1 Baseline characteristics of the study population.

| | | Outcomes | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Diabetes | Connective tissue diseases | Seropositive rheumatoid arthritis | Ulcerative colitis or Crohn disease | Crohn disease | Ulcerative colitis | Severe psychosis |
| All (*N*) | | 46,998 | 47,681 | 47,681 | 48,223 | 48,223 | 48,223 | 47,096 |
| Age | (29, 40] | 23,832 (50.7%) | 23,914 (50.2%) | 23,914 (50.2%) | 23,951 (49.7%) | 23,951 (49.7%) | 23,951 (49.7%) | 23,623 (50.2%) |
| | (40, 50] | 14,892 (31.7%) | 15,036 (31.5%) | 15,036 (31.5%) | 15,146 (31.4%) | 15,146 (31.4%) | 15,146 (31.4%) | 14,773 (31.4%) |
| | (50, 60] | 5,755 (12.2%) | 5,969 (12.5%) | 5,969 (12.5%) | 6,126 (12.7%) | 6,126 (12.7%) | 6,126 (12.7%) | 5,877 (12.5%) |
| | (60, 70] | 1,016 (2.2%) | 1,112 (2.3%) | 1,112 (2.3%) | 1,208 (2.5%) | 1,208 (2.5%) | 1,208 (2.5%) | 1,124 (2.4%) |
| | (70, Inf] | 1,503 (3.2%) | 1,650 (3.5%) | 1,650 (3.5%) | 1,792 (3.7%) | 1,792 (3.7%) | 1,792 (3.7%) | 1,699 (3.6%) |
| Gender | Male | 18,531 (39.4%) | 19,076 (40.0%) | 19,076 (40.0%) | 19,191 (39.8%) | 19,191 (39.8%) | 19,191 (39.8%) | 18,728 (39.8%) |
| | Female | 28,476 (60.6%) | 28,605 (60.0%) | 28,605 (60.0%) | 29,032 (60.2%) | 29,032 (60.2%) | 29,032 (60.2%) | 28,368 (60.2%) |
| Statin | No | 46,144 (98.2%) | 46,524 (97.6%) | 46,524 (97.6%) | 47,008 (97.5%) | 47,008 (97.5%) | 47,008 (97.5%) | 45,932 (97.5%) |
| | Yes | 854 (1.8%) | 1,157 (2.4%) | 1,157 (2.4%) | 1,215 (2.5%) | 1,215 (2.5%) | 1,215 (2.5%) | 1,164 (2.5%) |
| SES | Upper-level employees | 10,086 (21.5%) | 10,145 (21.3%) | 10,145 (21.3%) | 10,162 (21.1%) | 10,162 (21.1%) | 10,162 (21.1%) | 10,179 (21.6%) |
| | Self-employed or employers | 1,425 (3.0%) | 1,439 (3.0%) | 1,439 (3.0%) | 1,444 (3.0%) | 1,444 (3.0%) | 1,444 (3.0%) | 1,437 (3.1%) |
| | Lower-level employees | 15,254 (32.5%) | 15,315 (32.1%) | 15,315 (32.1%) | 15,416 (32.0%) | 15,416 (32.0%) | 15,416 (32.0%) | 15,398 (32.7%) |
| | Manual workers | 8,423 (17.9%) | 8,547 (17.9%) | 8,547 (17.9%) | 8,601 (17.8%) | 8,601 (17.8%) | 8,601 (17.8%) | 8,540 (18.1%) |
| | Unemployer | 3,993 (8.5%) | 4,087 (8.6%) | 4,087 (8.6%) | 4,124 (8.6%) | 4,124 (8.6%) | 4,124 (8.6%) | 4,007 (8.5%) |
| | Students | 1,486 (3.2%) | 1,487 (3.1%) | 1,487 (3.1%) | 1,500 (3.1%) | 1,500 (3.1%) | 1,500 (3.1%) | 1,439 (3.1%) |
| | Pensioners | 3,868 (8.2%) | 4,156 (8.7%) | 4,156 (8.7%) | 4,458 (9.2%) | 4,458 (9.2%) | 4,458 (9.2%) | 3,640 (7.7%) |
| | Unknown | 2,463 (5.2%) | 2,505 (5.3%) | 2,505 (5.3%) | 2,518 (5.2%) | 2,518 (5.2%) | 2,518 (5.2%) | 2,456 (5.2%) |
| No of teeth | 28–32 | 33,515 (71.3%) | 33,657 (70.6%) | 33,657 (70.6%) | 33,823 (70.1%) | 33,823 (70.1%) | 33,823 (70.1%) | 33,307 (70.7%) |
| | 24–27 | 9,173 (19.5%) | 9,378 (19.7%) | 9,378 (19.7%) | 9,522 (19.7%) | 9,522 (19.7%) | 9,522 (19.7%) | 9,267 (19.7%) |
| | 0–23 | 4,319 (9.2%) | 4,646 (9.7%) | 4,646 (9.7%) | 4,878 (10.1%) | 4,878 (10.1%) | 4,878 (10.1%) | 4,522 (9.6%) |
| I index | 0 | 12,290 (26.2%) | 12,584 (26.4%) | 12,584 (26.4%) | 12,813 (26.6%) | 12,813 (26.6%) | 12,813 (26.6%) | 12,430 (26.4%) |
| | 1–2 | 14,455 (30.8%) | 14,614 (30.6%) | 14,614 (30.6%) | 14,777 (30.6%) | 14,777 (30.6%) | 14,777 (30.6%) | 14,529 (30.8%) |
| | 3–4 | 8,664 (18.4%) | 8,764 (18.4%) | 8,764 (18.4%) | 8,840 (18.3%) | 8,840 (18.3%) | 8,840 (18.3%) | 8,655 (18.4%) |
| | >5 | 11,589 (24.7%) | 11,719 (24.6%) | 11,719 (24.6%) | 11,793 (24.5%) | 11,793 (24.5%) | 11,793 (24.5%) | 11,482 (24.4%) |
| D index | 0 | 18,511 (39.4%) | 18,621 (39.1%) | 18,621 (39.1%) | 18,845 (39.1%) | 18,845 (39.1%) | 18,845 (39.1%) | 18,573 (39.4%) |
| | 1–2 | 16,815 (35.8%) | 16,997 (35.6%) | 16,997 (35.6%) | 17,191 (35.6%) | 17,191 (35.6%) | 17,191 (35.6%) | 16,866 (35.8%) |
| | 3–4 | 6,222 (13.2%) | 6,351 (13.3%) | 6,351 (13.3%) | 6,420 (13.3%) | 6,420 (13.3%) | 6,420 (13.3%) | 6,231 (13.2%) |
| | >4 | 5,450 (11.6%) | 5,712 (12.0%) | 5,712 (12.0%) | 5,767 (12.0%) | 5,767 (12.0%) | 5,767 (12.0%) | 5,426 (11.5%) |
| DMF index | 0–13 | 13,401 (28.5%) | 13,463 (28.2%) | 13,463 (28.2%) | 13,477 (27.9%) | 13,477 (27.9%) | 13,477 (27.9%) | 13,309 (28.3%) |
| | 14–18 | 11,592 (24.7%) | 11,698 (24.5%) | 11,698 (24.5%) | 11,751 (24.4%) | 11,751 (24.4%) | 11,751 (24.4%) | 11,583 (24.6%) |
| | 19–23 | 11,168 (23.8%) | 11,333 (23.8%) | 11,333 (23.8%) | 11,448 (23.7%) | 11,448 (23.7%) | 11,448 (23.7%) | 11,229 (23.8%) |
| | >24 | 10,837 (23.1%) | 11,187 (23.5%) | 11,187 (23.5%) | 11,547 (23.9%) | 11,547 (23.9%) | 11,547 (23.9%) | 10,975 (23.3%) |
| CPI1 | 0–1 | 6,184 (13.2%) | 6,251 (13.1%) | 6,251 (13.1%) | 6,322 (13.1%) | 6,322 (13.1%) | 6,322 (13.1%) | 6,171 (13.1%) |
| | 2 | 30,177 (64.2%) | 30,388 (63.7%) | 30,388 (63.7%) | 30,676 (63.6%) | 30,676 (63.6%) | 30,676 (63.6%) | 30,127 (64.0%) |
| | 3–4 | 10,637 (22.6%) | 11,042 (23.2%) | 11,042 (23.2%) | 11,225 (23.3%) | 11,225 (23.3%) | 11,225 (23.3%) | 10,798 (22.9%) |
| Periodontitis | No | 35,030 (74.5%) | 35,436 (74.3%) | 35,436 (74.3%) | 35,817 (74.3%) | 35,817 (74.3%) | 35,817 (74.3%) | 35,013 (74.3%) |
| | Yes | 11,977 (25.5%) | 12,245 (25.7%) | 12,245 (25.7%) | 12,406 (25.7%) | 12,406 (25.7%) | 12,406 (25.7%) | 12,083 (25.7%) |
| Caries | No | 39,165 (83.3%) | 39,621 (83.1%) | 39,621 (83.1%) | 40,076 (83.1%) | 40,076 (83.1%) | 40,076 (83.1%) | 39,236 (83.3%) |
| | Yes | 7,842 (16.7%) | 8,060 (16.9%) | 8,060 (16.9%) | 8,147 (16.9%) | 8,147 (16.9%) | 8,147 (16.9%) | 7,860 (16.7%) |
| Apical periodontitis | No | 12,265 (26.1%) | 12,371 (25.9%) | 12,371 (25.9%) | 12,483 (25.9%) | 12,483 (25.9%) | 12,483 (25.9%) | 12,249 (26.0%) |
| | Yes | 34,733 (73.9%) | 35,310 (74.1%) | 35,310 (74.1%) | 35,740 (74.1%) | 35,740 (74.1%) | 35,740 (74.1%) | 34,847 (74.0%) |

Population sizes for each outcome include only non-prevalent individuals. Age groups are mutually exclusive, for example (29,40], means that interval is open on the left (29 not included) and closed on the right (40 included).

10.3389/froh.2022.956072

TABLE 2 The number of events, event rates per 10,000 person-years with 95% confidence intervals, and unadjusted and adjusted incidence rate ratios (IRR) with 95% confidence intervals.

| | | | Events | Incidence rate (1/10 000) | IRR, univariate | IRR, adjusted |
|---|---|---|---|---|---|---|
| Diabetes | N. teeth | 28–32 | 1,412 | 42.37 (40.19, 44.64) | (Reference) | (Reference) |
| | | 24–27 | 650 | 74.17 (68.57, 80.09) | 1.75 (1.60, 1.92) | 1.21 (1.09, 1.33) |
| | | 0–23 | 471 | 139.88 (127.53, 153.10) | 3.30 (2.97, 3.66) | 1.40 (1.22, 1.60) |
| | Periodontitis | No | 1,702 | 50.06 (47.71, 52.50) | (Reference) | (Reference) |
| | | Yes | 831 | 72.55 (67.70, 77.65) | 1.45 (1.33, 1.57) | 1.10 (1.01, 1.20) |
| | Caries | No | 1,949 | 51.39 (49.13, 53.72) | (Reference) | (Reference) |
| | | Yes | 584 | 77.60 (71.44, 84.16) | 1.51 (1.38, 1.66) | 1.12 (1.01, 1.23) |
| | Apical periodontitis | No | 478 | 40.48 (36.93, 44.28) | (Reference) | (Reference) |
| | | Yes | 2,055 | 61.09 (58.48, 63.79) | 1.51 (1.37, 1.67) | 1.16 (1.04, 1.30) |
| Connective tissue diseases | N. teeth | 28–32 | 338 | 9.97 (8.94, 11.10) | (Reference) | (Reference) |
| | | 24–27 | 129 | 14.07 (11.75, 16.72) | 1.41 (1.15, 1.73) | 1.21 (0.97, 1.50) |
| | | 0–23 | 43 | 11.36 (8.22, 15.30) | 1.14 (0.83, 1.56) | 1.00 (0.68, 1.47) |
| | Periodontitis | No | 372 | 10.67 (9.61, 11.81) | (Reference) | (Reference) |
| | | Yes | 138 | 11.52 (9.68, 13.61) | 1.08 (0.89, 1.31) | 1.09 (0.88, 1.34) |
| | Caries | No | 419 | 10.77 (9.76, 11.85) | (Reference) | (Reference) |
| | | Yes | 91 | 11.48 (9.24, 14.09) | 1.07 (0.85, 1.34) | 0.97 (0.76, 1.24) |
| | Apical periodontitis | No | 119 | 9.89 (8.19, 11.83) | (Reference) | (Reference) |
| | | Yes | 391 | 11.23 (10.15, 12.40) | 1.14 (0.93, 1.40) | 0.93 (0.73, 1.18) |
| Seropositive rheumatoid arthritis | N. teeth | 28–32 | 67 | 1.97 (1.53, 2.50) | (Reference) | (Reference) |
| | | 24–27 | 29 | 3.15 (2.11, 4.52) | 1.60 (1.03, 2.47) | 1.30 (0.81, 2.07) |
| | | 0–23 | 16 | 4.21 (2.41, 6.84) | 2.14 (1.24, 3.69) | 1.78 (0.89, 3.57) |
| | Periodontitis | No | 81 | 2.31 (1.84, 2.88) | (Reference) | (Reference) |
| | | Yes | 31 | 2.58 (1.75, 3.66) | 1.11 (0.74, 1.68) | 1.03 (0.66, 1.59) |
| | Caries | No | 91 | 2.33 (1.88, 2.86) | (Reference) | (Reference) |
| | | Yes | 21 | 2.64 (1.63, 4.03) | 1.13 (0.70, 1.82) | 1.01 (0.61, 1.68) |
| | Apical periodontitis | No | 29 | 2.40 (1.61, 3.45) | (Reference) | (Reference) |
| | | Yes | 83 | 2.37 (1.89, 2.94) | 0.99 (0.65, 1.51) | 0.77 (0.47, 1.27) |
| Ulcerative colitis and Crohn disease | N. teeth | 28–32 | 141 | 4.13 (3.48, 4.87) | (Reference) | (Reference) |
| | | 24–27 | 36 | 3.85 (2.70, 5.33) | 0.93 (0.65, 1.35) | 1.18 (0.80, 1.74) |
| | | 0–23 | 11 | 2.76 (1.38, 4.94) | 0.67 (0.36, 1.23) | 1.35 (0.63, 2.90) |
| | Periodontitis | No | 137 | 3.88 (3.26, 4.59) | (Reference) | (Reference) |
| | | Yes | 51 | 4.19 (3.12, 5.51) | 1.08 (0.78, 1.49) | 1.36 (0.97, 1.91) |
| | Caries | No | 159 | 4.03 (3.43, 4.71) | (Reference) | (reference) |
| | | Yes | 29 | 3.61 (2.41, 5.18) | 0.89 (0.60, 1.33) | 0.95 (0.62, 1.45) |
| | Apical periodontitis | No | 52 | 4.27 (3.19, 5.60) | (Reference) | (reference) |
| | | Yes | 136 | 3.85 (3.23, 4.56) | 0.90 (0.65, 1.24) | 0.89 (0.61, 1.31) |
| Crohn disease | N. teeth | 28–32 | 28 | 0.82 (0.54, 1.18) | (Reference) | (Reference) |
| | | 24–27 | 9 | 0.96 (0.44, 1.83) | 1.17 (0.55, 2.49) | 1.52 (0.68, 3.42) |
| | | 0–23 | 1 | 0.25 (0.01, 1.40) | 0.31 (0.04, 2.25) | 0.59 (0.06, 5.32) |
| | Periodontitis | No | 29 | 0.82 (0.55, 1.18) | (Reference) | (reference) |
| | | Yes | 9 | 0.74 (0.34, 1.40) | 0.90 (0.43, 1.90) | 1.12 (0.51, 2.45) |
| | Caries | No | 34 | 0.86 (0.60, 1.20) | (Reference) | (reference) |
| | | Yes | 4 | 0.50 (0.14, 1.27) | 0.58 (0.20, 1.63) | 0.57 (0.19, 1.71) |
| | Apical periodontitis | No | 14 | 1.15 (0.63, 1.93) | (Reference) | (reference) |
| | | Yes | 24 | 0.68 (0.43, 1.01) | 0.59 (0.31, 1.14) | 0.71 (0.32, 1.57) |

*(Continued)*

10.3389/froh.2022.956072

TABLE 2 Continued

| | | | Events | Incidence rate (1/10 000) | IRR, univariate | IRR, adjusted |
|---|---|---|---|---|---|---|
| Ulcerative colitis | N. teeth | 28–32 | 113 | 3.31 (2.73, 3.98) | (Reference) | (reference) |
| | | 24–27 | 27 | 2.89 (1.90, 4.20) | 0.87 (0.57, 1.33) | 1.10 (0.70, 1.71) |
| | | 0–23 | 10 | 2.51 (1.20, 4.61) | 0.76 (0.40, 1.45) | 1.53 (0.67, 3.48) |
| | Periodontitis | No | 108 | 3.06 (2.51, 3.69) | (Reference) | (reference) |
| | | Yes | 42 | 3.45 (2.49, 4.67) | 1.13 (0.79, 1.61) | 1.42 (0.98, 2.07) |
| | Caries | No | 125 | 3.17 (2.64, 3.78) | (Reference) | (reference) |
| | | Yes | 25 | 3.11 (2.01, 4.59) | 0.98 (0.64, 1.51) | 1.06 (0.67, 1.67) |
| | Endo. caries | No | 38 | 3.12 (2.21, 4.28) | (Reference) | (reference) |
| | | Yes | 112 | 3.17 (2.61, 3.82) | 1.02 (0.70, 1.47) | 0.96 (0.62, 1.49) |
| Severe psychosis | N. teeth | 28–32 | 28 | 0.82 (0.54, 1.18) | (Reference) | (reference) |
| | | 24–27 | 9 | 0.96 (0.44, 1.83) | 1.17 (0.55, 2.49) | 1.15 (0.93, 1.41) |
| | | 0–23 | 1 | 0.25 (0.01, 1.40) | 0.31 (0.04, 2.25) | 0.86 (0.60, 1.21) |
| | Periodontitis | No | 29 | 0.82 (0.55, 1.18) | (Reference) | (reference) |
| | | Yes | 9 | 0.74 (0.34, 1.40) | 0.90 (0.43, 1.90) | 1.04 (0.86, 1.25) |
| | Caries | No | 34 | 0.86 (0.60, 1.20) | (Reference) | (reference) |
| | | Yes | 4 | 0.50 (0.14, 1.27) | 0.58 (0.20, 1.63) | 1.12 (0.91, 1.38) |
| | Apical periodontitis | No | 14 | 1.15 (0.63, 1.93) | (Reference) | (reference) |
| | | Yes | 24 | 0.68 (0.43, 1.01) | 0.59 (0.31, 1.14) | 1.19 (0.95, 1.48) |

Adjusted using Poisson regression for sex, age, socioeconomic status, usage of statins in baseline, i-index, D-index, and CPI.

reimbursement. Our main finding was that oral health indices were related to diabetes but not to other chronic conditions. Thus, our findings support the close association of oral health, especially PD, to metabolic deterioration of glucose metabolism.

## Strengths and limitations of this study

The study population consisted of patients with at least one visit to dental healthcare in the City of Helsinki in 2 years period. This means that individuals without any dental visits were not eligible for the study. In principle, this non-eligible population includes people without any dental visits and those utilizing only the private sector dental healthcare. Usage of private healthcare may have caused some selection of the study population because about 36% of dental care was covered by it in the study period. As private care is most likely more commonly used by higher socioeconomic groups, this could imply that the selection process is drifted to lower socioeconomic groups. Still, as we had access to SES, we could also verify that higher SES were using the public health sector to a large extent (Table 1 and Supplementary Table 2). Among the potential confounders in this study were socio-demographic characteristics, such as age, sex, and SES status, available for the entire study population. The study was limited by the lack of information on smoking, alcohol use, and dietary habits, which may confound the findings because both are known risk factors for chronic diseases [30]. Our predecessor article found a strong positive association between periodontitis at baseline and subsequent risk of fatal pancreatic cancer. However, at the same time, we did not detect any association between periodontitis and lung cancer, which may be interpreted to indicate that confounding by smoking is probably not strong [32]. Altogether, additional studies are needed with more detailed measurements of confounders such as smoking and alcohol use to confirm these results. The diabetes diagnosis was based on the information reimbursement for the drug treatment for diabetes. This covers in practice all the subjects with drug treatment and the practice is to start medication already at the time of diagnosis [33]. However, it does not include the subjects with undiagnosed diabetes and asymptomatic hyperglycemia, and unfortunately HbA1c level information is not available from these registers. This is a weakness of the study, but if anything these shortcomings are likely to weaken the associations found in this study.

We also addressed statin medication, which certain but few studies have revealed with a low increased risk for the development of diabetes. Still, the risk is low both in absolute terms and when related to the reduction in coronary events. Clinical practice in patients with moderate or high cardiovascular risk or existing cardiovascular disease is well-documented [34], while statins are known to benefit the treatment and course of chronic periodontitis. Apart from their established LDL-cholesterol lowering effects, statins have shown additional secondary effects, including anti-inflammatory,

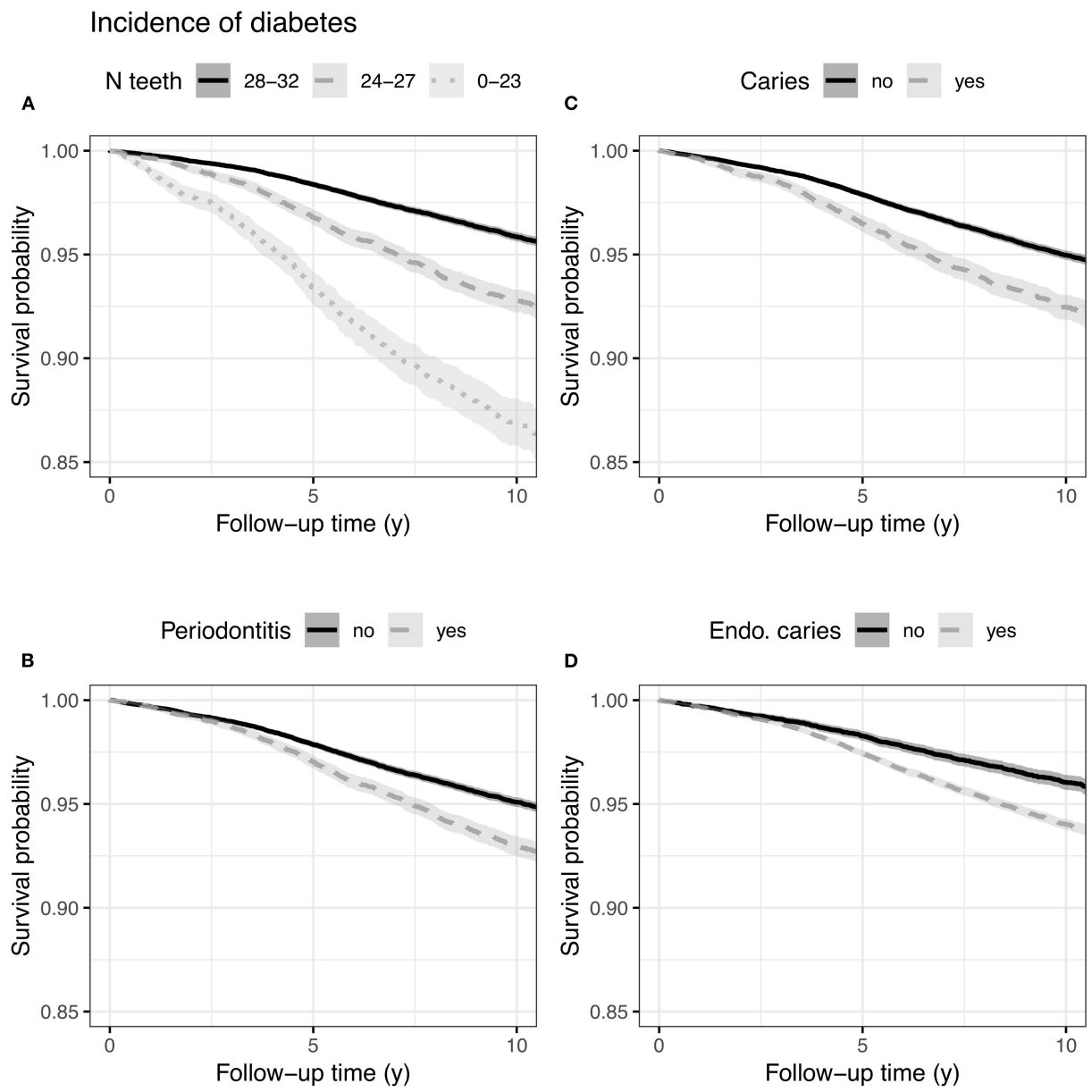



FIGURE 2
Incidence of diabetes and indicators of oral health. Kaplan–Meier curves with 95% confidence intervals. Number of teeth **(A)**, periodontitis **(B)**, caries **(C)**, endo/caries **(D)**.

immunomodulatory, antioxidant, antithrombotic, and endothelium stabilization effects, and promote angiogenesis [35]. Recent retrospective studies have demonstrated that patients with chronic periodontitis treated with simvastatin or atorvastatin had lower indexes of periodontitis than those not receiving statins [36, 37]. Atorvastatin can also restore endothelium-dependent vasodilation in normocholesterolemic cigarette smokers independent of changes in the lipids [38, 39]. Statins have recently been recorded to have beneficial effects on chronic periodontitis among smokers [40]. In our study, people with statin therapy were at higher risk of diabetes with IRR 2.49 (2.10-2.94). However, this number should be interpreted with great caution because it may contain the so-called "table two" fallacy. Table two fallacy is present when effects other than primary exposure are interpreted [41]. The main potential confounding factors between oral chronic infections and diabetes are tobacco, alcohol, socioeconomic status, age and sex, and genetic and dietary factors. Among the potential confounders in this study were socio-demographic characteristics, such as

TABLE 3 Incidence of diabetes, number needed to harm (NNH) based on the additive Poisson regression model.

| | | NNH (95% confidence interval) |
|---|---|---|
| N. teeth | 28–32 | (Reference) |
| | 24–27 | 1,004 (625, 2,561) |
| | 0–23 | 262 (190, 420) |
| Periodontitis | No | (Reference) |
| | Yes | 1,736 (903, 22,103) |
| Caries | No | (Reference) |
| | Yes | 1,342 (709, 12,497) |
| Apical periodontitis | No | (Reference) |
| | Yes | 2,052 (1,029, 349,261) |

Adjusted for gender, age, socioeconomic status, usage of statins in baseline, i-index, D-index, and CPI.

age, sex, and socio-economic status, available for the entire study population.

We measured our outcomes as starting of new special reimbursement. This means that the diagnosis of the condition has taken place earlier. This lag may vary between the diagnosis and between individuals considerably. This means that results may be biased if the lag between diagnosis and the start of reimbursement is affected by oral health status. We tried to control this source of bias by starting a follow-up after 2 years of the first dental visit recorded. We could assess the type of diabetes only by the kind of therapy. However, those treated only with insulin comprise type 1 diabetic patients and some long-standing type 2 diabetic patients whose disease can be classified as insulin-requiring. The demarcation between specific types of new-onset diabetes in adults in individual cases may also be somewhat arbitrary in clinical practice. It is also possible regarding diabetic subjects treated with lifestyle only. However, as lifestyle interventions are difficult to implement and may delay unnecessarily the start of drug treatment, the Finnish Current Care guideline (original version published in 2007 and updated several times) recommended that drug treatment with metformin should be initiated if not contraindicated concomitantly with lifestyle interventions [42]. We have previously shown that the implementation of these guidelines has been successful, [43] and thus, nearly all the subjects with a clinically verified diagnosis of diabetes were included making this population representative.

The main strength of the exposure measurements is that they contained detailed clinical information about oral health and dental procedures. Our primary exposures—periodontal, cariological status, and apical periodontitis—were determined by procedure codes, which means that the number of false positives is very low. Studies investigating the association between periodontal disease and diabetes and other chronic diseases have used various measures to define the periodontal disease and how disease progression is ascertained. There is no standardized definition or clinical criteria for periodontal disease in periodontal epidemiological research, hindering comparisons of studies examining the association between periodontal disease and chronic diseases [44]. Periodontal disease is generally diagnosed by probing and measuring alveolar bone height with radiographs. In this study, we used the information on dental status presented by the number of teeth. Dental infections, caries, or periodontitis can potentially be assumed as the reason for the extracted teeth. Earlier studies have hypothesized that missing teeth reflect an individual lifetime accumulation of oral inflammation. In the national FINRISK 1997 study Finnish population-based survey of 8,446 subjects with 13 years of follow-up, Liljestrand et al. revealed that missing teeth predict incident cardiovascular events, diabetes, and death, and periodontitis was the main cause of tooth loss in the middle-aged and elderly [45]. While, other studies like Chauncey et al. and Jovino-Silvera et al. showed caries complications to be the primary reason for tooth extraction. In these studies, the size of the studied population is less than one thousand [46, 47]. Caries can lead to dental pulp necrosis with subsequent infection spread in the apical area and beyond. It can leave chronic inflammation to persist in the apical area, apical periodontitis [48].

In this study, we used the division into three teeth groups due to a lack of consensus. The groups with the number of teeth overlap with each other concerning the evident causes due to which individual teeth are extracted, but hypothetical cause grouping may aid in the interpretation of results: the first group with teeth 28 to 32 represent periodontally healthy subjects or lack of or extractions of third molars; those with teeth 24 to 27 may have additionally lost them due to orthodontic reasons, periodontitis, or caries; and those with teeth 0 to 23 most likely suffer from chronic periodontitis [45]. Thus, these groups with fewer teeth eventually represent ongoing or treated advanced oral disease with a plausible systemic inflammatory burden.

We used reported history of procedure codes and dental status represented by the number of teeth, oral health indices, initial caries, decayed/missing/filled teeth, and need for periodontal treatment according to the involvement of gingival pockets. Furthermore, we defined periodontitis as a binary variable (no/yes) based on the procedure codes of periodontitis treatment in the years 2001 and 2002, when we collected data on patients' oral health status. Overall, the collected data support the association between periodontitis, caries, apical periodontitis, and diabetes.

## Comparisons with other studies

Many previous studies investigated biological connections between periodontitis and diabetes-focused on the impact of diabetes on periodontal pathogenesis. There is evidence for

the bidirectional connection between these two diseases with associated feedback effects. A dysregulated immune system is essential to the pathogenesis of diabetes and its complications. Systemic changes in cytokine and matrix metalloproteinase (MMP) levels impact the pathogenesis of type 2 diabetes, associated with physiological, nutritional, and metabolic changes, including hyperglycemia, production of advanced glycation end-products (AGE), hyperlipidemia, and increased adiposity [44, 49]. These mechanisms can affect by weakening the individual's immune response and periodontal condition. Proteins are glycated and eventually converted to AGE products in persistent hyperglycemia. These irreversible glycation processes of proteins have several consequences, including immune and proinflammatory dysregulation manifested by a pronounced, long-lasting inflammatory state and weakened self-limitation resolution of immune responses [44, 49]. These processes mediate pathophysiological mechanisms promoting the development and progression of periodontitis in diabetes, interfering with the physiologic tissue repair and wound healing. When AGE products bind to signaling receptors of several cell types, one of the results is the production and release of reactive oxygen species, proinflammatory mediators, and MMPs. The reactive oxygen species, cytokines, and proteases promote inflammation and ultimately exacerbate periodontal tissue destruction through an exaggerated inflammatory response and limited tissue repair [50]. Concerning the potential impact of periodontitis on the disease processes of diabetes, there is little biological evidence available. The periodontal microbiota appears unaltered by diabetes, and there is little evidence that it may influence glycemic control. The systemic inflammation triggered by periodontitis can affect the regulation of the serum glucose level through an increase in the levels of inflammatory mediators, such as tumor necrosis factor-alpha and interleukin-6, MMPs, oxygen radicals, and acute-phase proteins, which interfere with the glucose control mechanism, inhibit and inactivate the insulin receptors, and reduce the uptake of glucose into the cell. In the presence of severe periodontitis, serum glucose levels can become elevated over the years in a clinically significant manner, even without diabetes. MMP-8 can proteolytically process insulin receptors [11]. If diabetes is already present with the simultaneous presence of untreated severe periodontitis, proper glycemic control is probably more challenging, and the risk for diabetes complications is increased [51].

Some former studies have found that the risk of systemic disease can be decreased with periodontal therapy. The various clinical trials demonstrated that periodontal treatment prevented or modified the progression of systemic diseases. All of the studies conducted were limited by a small sample size and inconsistent outcome measures across studies, and the limited duration of follow-up [52, 53]. In Sabharwal et al.'s review article, the majority of 23 randomized clinical trials revealed consistent and moderate effects of periodontal treatment on serum glycemic control in type 2 diabetic individuals. The treatment of periodontitis may thus contribute to improvements in the mouth and throughout the body, with a reduction of the concentrations of inflammatory mediators and MMPs in the blood resulting in the reduction of the average serum glucose levels and improvements in the demonstrated lipid profiles, in general, improving the control of diabetes [52]. A recent study by D'Aiuto et al. showed that periodontitis treatment reduced 0.6% HbA1c in patients ($N = 264$) with type 2 diabetes and moderate-to-severe periodontitis after 12 months [13].

This study comprises a large unselected population with representative cohorts of patients with periodontitis and apical periodontitis followed up over 10 years. Despite the limitations inherent in this type of study, the results are likely to be generalizable to similar populations of individuals with chronic oral diseases. The association exists between chronic oral diseases and diabetes, which warrants close collaboration among each patient's healthcare professionals, especially among medical and dental care providers.

## Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

## Ethics statement

Written informed consent was not required for this study in accordance with the local legislation and institutional requirements.

## Author contributions

All authors participated in the data interpretation and the manuscript's critical revision. All authors exerted full access to all data (including programming code, statistical reports, and tables) during the study and are responsible for the data integrity and data analysis accuracy. All authors contributed to the article and approved the submitted version.

## Funding

The work was supported by grants from the Finnish Dental Association, Finnish Outpatient Research Foundation, the University of Helsinki Tulevaisuusrahasto, the Helsinki University Hospital Research Foundation, the Apollonia Foundation Helsinki, Finland, and Karolinska Institutet, Stockholm, Sweden. The funders exerted no roles in study design, data collection analysis, interpretation, or report.

## Acknowledgments

The authors gratefully acknowledge the leading dentist Merja Auero, the Public Dental Service of the City of Helsinki, and dentist Tuomo Maisala, technical assistant collecting the data from the patient register of the Public Dental Service of the City of Helsinki. We also thank Statistics Finland, the Finnish Social Insurance Institution, and the Finnish Cancer Registry.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/froh.2022.956072/full#supplementary-material

## References

1. Manji F, Dahlen G, Fejerskov O. Caries and periodontitis: contesting the conventional wisdom on their aetiology. *Caries Res.* (2018) 52:548–64. doi: 10.1159/000488948

2. Borgnakke WS, Ylöstalo PV, Taylor GW, Genco RJ. Effect of periodontal disease on diabetes: systematic review of epidemiologic observational evidence. *J Periodontol.* (2013) 84:S135–52. doi: 10.1902/jop.2013.1340013

3. Papageorgiou Spyridon N., Hagner M, Nogueira AVB, Franke A, Jäger A, Deschner J. Inflammatory bowel disease and oral health: systematic review and a meta-analysis. *J Clin Periodontol.* (2017) 44:382–93. doi: 10.1111/jcpe.12698

4. Grover V, Kapoor A, Sehgal K, Kaur G. Chronic inflammation and carcinogenesis – emerging role of chronic inflammatory periodontal disease. *Cancer Res Front.* (2016) 2:200–25. doi: 10.17980/2016.200

5. Desvarieux M, Demmer RT, Rundek T, Boden-Albala B, Jacobs DR Jr, Sacco RL, et al. Periodontal microbiota and carotid intima-media thickness: the oral infections and vascular disease epidemiology study (INVEST). *Circulation.* (2005) 111:576–82. doi: 10.1161/01.CIR.0000154582.37101.15

6. Lockhart PB, Bolger AF, Papapanou PN, Osinbowale O, Trevisan M, Levison ME, et al. Periodontal disease and atherosclerotic vascular disease: does the evidence support an independent association? A scientific statement from the American Heart Association. *Circulation.* (2012) 125:2520–44. doi: 10.1161/CIR.0b013e31825719f3

7. Tonetti MS, Van Dyke TE. Periodontitis and atherosclerotic cardiovascular disease: consensus report of the Joint EFP/AAPWorkshop on periodontitis and systemic diseases. *J Periodonto.* (2013) 84:S24–9. doi: 10.1902/jop.2013.1340019

8. Segura-Egea JJ, Martín-González J, Castellanos-Cosano L. Endodontic medicine: connections between apical periodontitis and systemic diseases. *Int Endodontic J.* (2015) 48:933–51. doi: 10.1111/iej.12507

9. Aminoshariae A, Kulild JC, Fouad AF. The impact of endodontic infections on the pathogenesis of cardiovascular disease(s): a systematic review with meta-analysis using GRADE. *J Endodontics.* (2018) 44:1361–6.e3. doi: 10.1016/j.joen.2018.06.011

10. Duncan BB, Schmidt MI. The epidemiology of low-grade chronic systemic inflammation and type 2 diabetes. *Diabetes Technol Therapeut.* (2006) 8:7–17. doi: 10.1089/dia.2006.8.7

11. Lauhio A, Färkkilä E, Pietiläinen KH, Åström P, Winkelmann A, Tervahartiala T, et al. Association of MMP-8 with obesity, smoking and insulin resistance. *Eur J Clin Invest.* (2016) 46:757–65. doi: 10.1111/eci.12649

12. Lalla E, Papapanou PN. Diabetes mellitus and periodontitis: a tale of two common interrelated diseases. *Nat Rev Endocrinol.* (2011) 7:738–48. doi: 10.1038/nrendo.2011.106

13. D'Aiuto F, Gkranias N, Bhowruth D, Khan T, Orlandi M, Suvan J, et al. Systemic effects of periodontitis treatment in patients with type 2 diabetes: a 12 month, single-centre, investigator-masked, randomised trial. *Lancet Diabetes Endocrinol.* (2018) 6:954–65. doi: 10.1016/S2213-8587(18)30038-X

14. Kaur S, White S, Bartold PM. Periodontal disease and rheumatoid arthritis: a systematic review. *J Dent Res.* (2013) 92:399–408. doi: 10.1177/0022034513483142

15. Koziel J, Mydel P, Potempa J. The link between periodontal disease and rheumatoid arthritis: an updated review. *Curr Rheumatol Rep.* (2014) 16:408. doi: 10.1007/s11926-014-0408-9

16. Araújo VMA, Melo IM, Lima V. Relationship between periodontitis and rheumatoid arthritis: review of the literature. mediators of inflammation. *Mediators Inflamm.* (2015) 2015:259074. doi: 10.1155/2015/259074

17. Ramamurthy NS, Greenwald RA, Celiker MY, Shi EY. Experimental arthritis in rats induces biomarkers of periodontitis which are ameliorated by gene therapy with tissue inhibitor of matrix metalloproteinases. *J Periodontol.* (2005) 76:229–33. doi: 10.1902/jop.2005.76.2.229

18. Cantley MD, Haynes DR, Marino V, Bartold PM. Pre-existing periodontitis exacerbates experimental arthritis in a mouse model. *J Clin Periodontol.* (2011) 38:532–41. doi: 10.1111/j.1600-051X.2011.01714.x

19. Statistics Finland. *Health—Causes of Death.* (2016). http://www.stat.fi/til/ksyyt/index_en.html (accessed November 7, 2016).

20. Social Insurance Institution of Finland Kela. *Overview of Benefit Programmes 2007.* Finland: Social Insurance Institution (2007).

21. WHO. *International Classification of Diseases for Oncology*. 3rd ed (ICD-O-3) (2014). Available online at: https://www.who.int/standards/classifications/other-classifications/international-classification-of-diseases-for-oncology (accessed February 25, 2015).

22. Teppo L, Pukkala E, Lehtonen M. Data quality and quality control of a population-based cancer registry. Experience in Finland. *Acta Oncol.* (1994) 33:365–369. 8018367

23. Korhonen P, Malila N, Pukkala E, Teppo L, Albanes D, Virtamo J. The Finnish Cancer Registry as follow-up source of a large trial cohort–accuracy and delay. *Acta Oncol.* (2002) 41:381–8. doi: 10.1080/028418602760169442

24. Holmes MD, Chen WY, Feskanich D, Kroenke CH, Colditz GA. Physical activity and survival after breast cancer diagnosis. *JAMA.* (2005) 293:2479–86. doi: 10.1001/jama.293.20.2479

25. Ballard-Barbash R, Friedenreich CM, Courneya KS, Siddiqi SM, McTiernan A, Alfano CM. Physical activity, biomarkers, and disease outcomes in cancer survivors: a systematic review. *J Natl Cancer Inst.* (2012) 104:815–40. doi: 10.1093/jnci/djs207

26. Prince MJ, Wu F, Guo Y, Gutierrez Robledo LM, O'Donnell M, Sullivan R, et al. The burden of disease in older people and implications for health policy and practice. *Lancet.* (2015) 385:549–62. doi: 10.1016/S0140-6736(14)61347-7

27. Nilsson MI, Bourgeois JM, Nederveen JP, Leite MR, Hettinga MP, Bujak AL, et al. Lifelong aerobic exercise protects against inflammaging and cancer. *PLoS ONE.* (2019) 14:e0210863. doi: 10.1371/journal.pone.0210863

28. Lipsitch M, Tchetgen E, Cohen T. Negative controls: a tool for detecting confounding and bias in observational studies. *Epidemiology.* (2010) 21:383–8. doi: 10.1097/EDE.0b013e3181d61eeb

29. Ainamo J, Barmes D, Beagrie G, Cutress T, Martin J, Sardo-Infirri J. Development of the World Health Organization (WHO) community periodontal index of treatment needs (CPITN). *Int Dent J.* (1982) 32:281–91.

30. World Health Organization. *Oral Health Surveys : Basic Methods.* (1997). Available online at: https://apps.who.int/iris/bitstream/handle/10665/97035/9789241548649_eng.pdf?sequence=1&isAllowed=y (accessed November 7, 2016).

31. R Core Team. *R: A Language and Environment for Statistical Computing.* Vienna: R Foundation for Statistical Computing (2016). Available online at: https://www.R-project.org/

32. Heikkilä P, But A, Sorsa T, Haukka J. Periodontitis and cancer mortality: register-based cohort study of 68,273 adults in 10-year follow-up. *Int J Cancer.* (2018) 142:2244–53. doi: 10.1002/ijc.31254

33. Niskanen L, Partonen T, Auvinen A, Haukka J. Trends and predictors in all-cause and cause-specific mortality in diabetic and reference populations during 21 years of follow-up. *J Epidemiol Community Health.* (2020) 74:950–6. doi: 10.1136/jech-2019-213602

34. Sattar N, Preiss D, Murray HM, Welsh P, Buckley BM, de Craen AJ, et al. Statins and risk of incident diabetes: a collaborative meta-analysis of randomised statin trials. *Lancet.* (2010) 375:735–42. doi: 10.1016/S0140-6736(09)61965-6

35. Landmesser U, Bahlmann F, Mueller M, Spiekermann S, Kirchhoff N, Schulz S, et al. Simvastatin versus ezetimibe. *Circulation.* (2005) 111:2356–63. doi: 10.1161/01.CIR.0000164260.82417.3F

36. Lindy O, Suomalainen K, Mäkelä M, Lindy S. Statin use is associated with fewer periodontal lesions: a retrospective study. *BMC Oral Health.* (2008) 8:16. doi: 10.1186/1472-6831-8-16

37. Saxlin T, Suominen-Taipale L, Knuuttila M, Alha P, Ylöstalo P. Dual effect of statin medication on the periodontium. *J Clin Periodontol.* (2009) 36:997–1003. doi: 10.1111/j.1600-051X.2009.01484.x

38. Beckman JA, Liao JK, Hurley S, Garrett LA, Chui D, Mitra D, et al. Atorvastatin restores endothelial function in normocholesterolemic smokers independent of changes in low-density lipoprotein. *Circ Res.* (2004) 95:217–23. doi: 10.1161/01.RES.0000134628.96682.9b

39. Agewall S, Hernberg Å. Atorvastatin normalizes endothelial function in healthy smokers. *Clin Sci.* (2006) 111:87–91. doi: 10.1042/CS20060033

40. Kumari M, Martande SS, Pradeep AR. Subgingivally delivered 1.2% atorvastatin in the treatment of chronic periodontitis among smokers: a randomized, controlled clinical trial. *J Investig Clin Dentist.* (2017) 8:e12213. doi: 10.1111/jicd.12213

41. Westreich D, Greenland S. The table 2 fallacy: presenting and interpreting confounder and modifier coefficients. *Am J Epidemiol.* (2013) 177:292–8. doi: 10.1093/aje/kws412

42. *Working Group Appointed by the Finnish Medical Society Duodecim, the Finnish Society of Internal Medicine and the Medical Advisory Board of the Finnish Diabetes Society. Current Care Guideline Type 2 Diabetes 1 and Insulin-Deficient Diabetes.* (2019). Available online at: http://www.kaypahoito.fi/web/english/guidelineabstracts/guideline?id=ccs00032 (accessed March 11, 2019).

43. Niskanen L, Hahl J, Haukka J, Leppä E, Miettinen T, Mushnikov V, et al. Type 2 diabetes and treatment intensification in primary care in Finland. *Acta Diabetol.* (2018) 55:1171–9. doi: 10.1007/s00592-018-1199-7

44. Papapanou PN. Periodontal diseases: epidemiology. *Ann Periodontol.* (1996) 1:1–36. doi: 10.1902/annals.1996.1.1.1

45. Liljestrand JM, Havulinna AS, Paju S, Männistö S, Salomaa V, Pussinen PJ. Missing teeth predict incident cardiovascular events, diabetes and death. *J Dental Res.* (2015) 94:1055–62. doi: 10.1177/0022034515586352

46. Chauncey HH, Glass RL, Alman JE. Dental caries. principal cause of tooth extraction in a sample of US male adults. *Caries Res.* (1989). 23:200–5. doi: 10.1159/000261178

47. Jovino-Silvera RC, de Franca Caldas A, de Souza EHA, Gusmao ES. Primary reason for tooth extraction in a Brazilian adult population. *Oral Health Prev Dent.* (2005) 3:151-7.

48. Nair PNR. Pathogenesis of apical periodontitis and the causes of endodontic failures. *Crit Rev Oral Biol Med.* (2004) 15:348–81. doi: 10.1177/154411130401500604

49. Ryan ME, Ramamurthy NS, Sorsa T, Golub LM. MMP-mediated events in diabetes. *Ann N Y Acad Sci.* (1999) 878:311–34. doi: 10.1111/j.1749-6632.1999.tb07692.x

50. Katz J, Wallet S, Cha S. Periodontal disease and the oral-systemic connection: "is it all the RAGE?" *Quintessence Int.* (2010) 41:229–37.

51. Batty GD, Li Q, Huxley R, Zoungas S, Taylor BA, Neal B, et al. Oral disease in relation to future risk of dementia and cognitive decline: prospective cohort study based on the Action in Diabetes and Vascular Disease: preterax and Diamicron Modified-Release Controlled Evaluation (ADVANCE) trial. *Eur Psychiatry.* (2013) 28:49–52. doi: 10.1016/j.eurpsy.2011.07.005

52. Sabharwal A, Gomes-Filho IS, Stellrecht E, Scannapieco FA. Role of periodontal therapy in management of common complex systemic diseases and conditions: an update. *Periodontology.* (2018) 78:212–26. doi: 10.1111/prd.12226

53. Rodríguez-Medina C, Agudelo-Suárez AA, Botero JE. Weak evidence hinders the understanding of the benefits of periodontal therapy on glycemic control in patients with diabetes and periodontitis. *J Evid Based Dental Pract.* (2016) 16:236–8. doi: 10.1016/j.jebdp.2016.11.005

frontiers | Frontiers in Dental Medicine

TYPE Original Research
PUBLISHED 25 November 2022
DOI 10.3389/fdmed.2022.992722



OPEN ACCESS

EDITED BY
Bjorn Olsen,
Harvard Medical School, United States

REVIEWED BY
Takehito Ouchi,
Tokyo Dental College, Japan
Zhipeng Fan,
Capital Medical University, China

*CORRESPONDENCE
Ellen Heber-Katz
heberkatz@icloud.com

†PRESENT ADDRESSES
Elan Zebrowitz,
New York Medical College, Valhalla, NY, United States
Sam Bollinger,
Cancer Biology Graduate Group, Stanford, CA, United States
Yong Zhang,
Analytical Research & Development, Merck & Co, Kenilworth, NJ, Uniited States
Jing Cheng,
Alcon Laboratories, Duluth, GA, United States

‡These authors share first authorship

SPECIALTY SECTION
This article was submitted to Regenerative Dentistry, a section of the journal Frontiers in Dental Medicine

RECEIVED 12 July 2022
ACCEPTED 20 October 2022
PUBLISHED 25 November 2022

CITATION
Zebrowitz E, Aslanukov A, Kajikawa T, Bedelbaeva K, Bollinger S, Zhang Y, Sarfatti D, Cheng J, Messersmith PB, Hajishengallis G and Heber-Katz E (2022) Prolyl-hydroxylase inhibitor-induced regeneration of alveolar bone and soft tissue in a mouse model of periodontitis through metabolic reprogramming.
Front. Dent. Med 3:992722.
doi: 10.3389/fdmed.2022.992722



# Prolyl-hydroxylase inhibitor-induced regeneration of alveolar bone and soft tissue in a mouse model of periodontitis through metabolic reprogramming

Elan Zebrowitz[1†‡], Azamat Aslanukov[1‡], Tetsuhiro Kajikawa[2], Kamila Bedelbaeva[1], Sam Bollinger[1†], Yong Zhang[1†], David Sarfatti[1], Jing Cheng[3†], Phillip B. Messersmith[3,4], George Hajishengallis[2] and Ellen Heber-Katz[1*]

[1]Lankenau Institute for Medical Research, Wynnewood, PA, United States, [2]Department of Basic and Translational Sciences, University of Pennsylvania School of Dental Medicine, Philadelphia, PA, United States, [3]Materials Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, United States, [4]Department of Bioengineering and Materials Science and Engineering, UC Berkeley, Berkeley, CA, United States

Bone injuries and fractures reliably heal through a process of regeneration with restoration to original structure and function when the gap between adjacent sides of a fracture site is small. However, when there is significant volumetric loss of bone, bone regeneration usually does not occur. In the present studies, we explore a particular case of volumetric bone loss in a mouse model of human periodontal disease (PD) in which alveolar bone surrounding teeth is permanently lost and not replaced. This model employs the placement of a ligature around the upper second molar for 10 days leading to inflammation and bone breakdown and closely replicates the bacterially-induced inflammatory etiology of human PD to induce bone degeneration. After ligature removal, mice are treated with a timed-release formulation of a small molecule inhibitor of prolylhydroxylases (PHDi; 1,4-DPCA) previously shown to induce epimorphic regeneration of soft tissue in non-regenerating mice. This PHDi induces high expression of HIF-1α and is able to shift the metabolic state from OXPHOS to aerobic glycolysis, an energetic state used by stem cells and embryonic tissue. This regenerative response was completely blocked by *siHIF1a.* In these studies, we show that timed-release 1,4-DPCA rapidly and completely restores PD-affected bone and soft tissue with normal anatomic fidelity and with increased stem cell markers due to site-specific stem cell migration and/or de-differentiation of local tissue, periodontal ligament (PDL) cell proliferation, and increased vascularization. In-vitro studies using gingival tissue show that 1,4-DPCA indeed induces de-differentiation and the expression of stem cell markers but does not exclude the role of migrating stem cells. Evidence of metabolic

Abbreviations
B6, C57BL/6 mouse; CEJ, cemento-enamel junction; DPCA, 1,4-dihydrophenonthrolin-4-one-3-carboxylic acid; *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase: *Glut-1*,glucose transporter type 1; HIF-1α; hypoxia-inducible factor-1alpha; *Ldh-a*, lactate dehydrogenase a; MRL, Murphy Roths Large mouse; *Pdk1*, pyruvate dehydrogenase kinase; PD, periodontal disease; PDL, periodontal ligament; *Pgk1*, phosphogylcerate kinase 1; PHDi, prolyl hydroxylase inhibitor.

reprogramming is seen by the expression of not only HIF-1α, its gene targets, and resultant de-differentiation markers, but also the metabolic genes *Glut-1, Gapdh, Pdk1, Pgk1* and *Ldh-a* in the periodontal tissue.

KEYWORDS

alveolar bone, GAPDH, GLUT-1, HIF-1α, ldh-a: micro-CT, mice, oct3/4, periodontal disease, periodontal ligament, PGK1, PDK1, PHDI, pulp, 1, 4-DPCA

## Introduction

Humans, like virtually all mammals, heal tissue and organ injuries by the process of scarring with limited restoration of normal anatomical integrity and functionality. This is in contrast to species such as newts, salamanders and other vertebrates which heal perfectly through the process of regeneration. In these examples, bone and soft tissues are replaced creating indistinguishable replicas of lost or damaged tissues (1–3). There are several routes to mammalian regeneration being actively considered for regenerative therapies, usually involving the use of stem cells with or without bioscaffolds (4–9). In a dental context, pulp stem cells as an autologous graft and the use of compounds such as high molecular weight hyaluronic acid can affect periodontal bone healing (10–12). However, the possibility of a systemically-acting drug which alone could induce regeneration in multiple tissues would be an intriguing therapy.

The path to identifying such a drug began with the serendipitous observation that the MRL mouse strain, long employed in autoimmunity studies, possessed an unusual capacity for tissue regeneration. Through-and-through ear pinna wounds used as life-long mouse identifiers healed by fully closing without scarring within 30 days. All tissue types found in the ear including epidermis, dermis, blood vessels, nerve, glands, cartilage and hair follicles were restored (13, 14). Furthermore, multiple studies showed that this regenerative phenotype extended to MRL cornea, tendon, cartilage, muscle, fat, and other tissues (15–18). However, bone injuries were largely unexplored.

Insight into the biological basis of the regenerative phenotype of the MRL mouse came from the fact that the adult MRL employs a strongly aerobic glycolytic metabolism in the basal state also seen in embryos and stem cell niches. This metabolic state was enhanced during regenerative wound healing but not normal wound repair (16, 19, 20). One well-known molecule that regulates aerobic glycolysis is hypoxia-inducible factor (HIF-1α; 21, 22) which was highly up-regulated in the MRL upon initiation of injury (23). Blocking HIF-1α using *siHIF1a* completely blocked the regenerative response (23).

The term "metabolic reprogramming" has been generally used to describe the metabolic state of tumor cells relative to normal cells (24–27). However, it has also been used to describe changes in metabolism necessary for proliferating cells in general. Here, glycolysis, with increased lactic acidosis, glucose consumption, and amino acid and nucleic acid synthesis, is preferred over oxidative phosphorylation. By transiently increasing the level of HIF-1α, a major positive regulator of aerobic glycolysis, through blocking prolyl hydroxylases (PHDs) that negatively control HIF-1α levels, the PHD inhibitor (PHDi), 1,4-DPCA, was employed as a regeneration activator.

In the current set of studies, we explored the possibility that 1,4-DPCA, (28) could also have a positive effect on the regeneration of bone and soft tissue of the jaw in mice. The use of 1,4-DPCA delivered in a hydrogel or coupled to PEG was previously shown by our laboratories to lead to a regenerative healing response resulting in closure of ear holes and replacement of soft tissue in mice (16, 23, 29) indistinguishable from that seen in MRL mice. This outcome was also seen in a second soft tissue target, enhanced DPCA-induced liver regeneration (30). We employed a mouse model of periodontal disease induced by placing a ligature around the upper second molar for 10 days, leading to an oral bacterial accumulation and bone degeneration, followed by removal of that ligature (31–33). 1,4-DPCA coupled to a PEG gel was then administered systemically followed by a second dose of drug administered 8 days later. After 20 days post-ligature removal and administration of drug, we saw full replacement of alveolar bone and gingival soft tissue.

Using Micro-CT analysis of bone damage and regeneration combined with immunohistochemistry and gene expression, we explored the DPCA effect (23, 29) for over 220 days in longitudinal studies to determine if full regeneration was achieved and maintained. In addition to the rapid recovery of alveolar bone architecture and gingival soft tissue there was significant regeneration of periodontal ligament (PDL) which attaches tooth to bone, and in the pulp of the teeth. Here, we found increased cell proliferation, HIF-1α levels, bone-PDL interactions, vascularization, and stem cell markers. These included 1) scleraxis, a transcription factor expressed in tendon progenitor populations, mature tendon and PDL fibroblasts (34–37) and 2) neurofilament, a structural protein of mature nerve fibers, seen in the pulp and PDL (38). The known pulp markers of progenitor cell populations shown to be mesenchymal stem cells (MSC), alpha smooth muscle actin (aSMA) and CD44 (39–42), were also increased after drug treatment. Furthermore, the possibility of tissue de-differentiation followed by re-differentiation into mature

tissue, a hallmark of classic epimorphic regeneration with the expression of Oct3/4, Nanog, Pax7, Sox2, and CD34, was supported by these results and had previously been noted in ear pinna regeneration (1–3, 23, 29).

In the broadest sense, bone is the biological "bioscaffold" which structurally supports the soft tissues of the body. The ability to regenerate bone through the up-regulation of HIF-1α by a systemically acting drug extends the range of possibilities of regenerative therapies.

# Results

## Ligature-induced bone degeneration

Micro-CT scanning was performed on C57BL/6 (B6) female mice 2 days prior to placement of ligature (day minus 2) around the upper (maxillary) left 2nd molar (**Figure 1Aa,b**, ref. 31). Mice were re-scanned on day 5 (with ligature present) and again on day 10 (immediately after ligature removal). Image

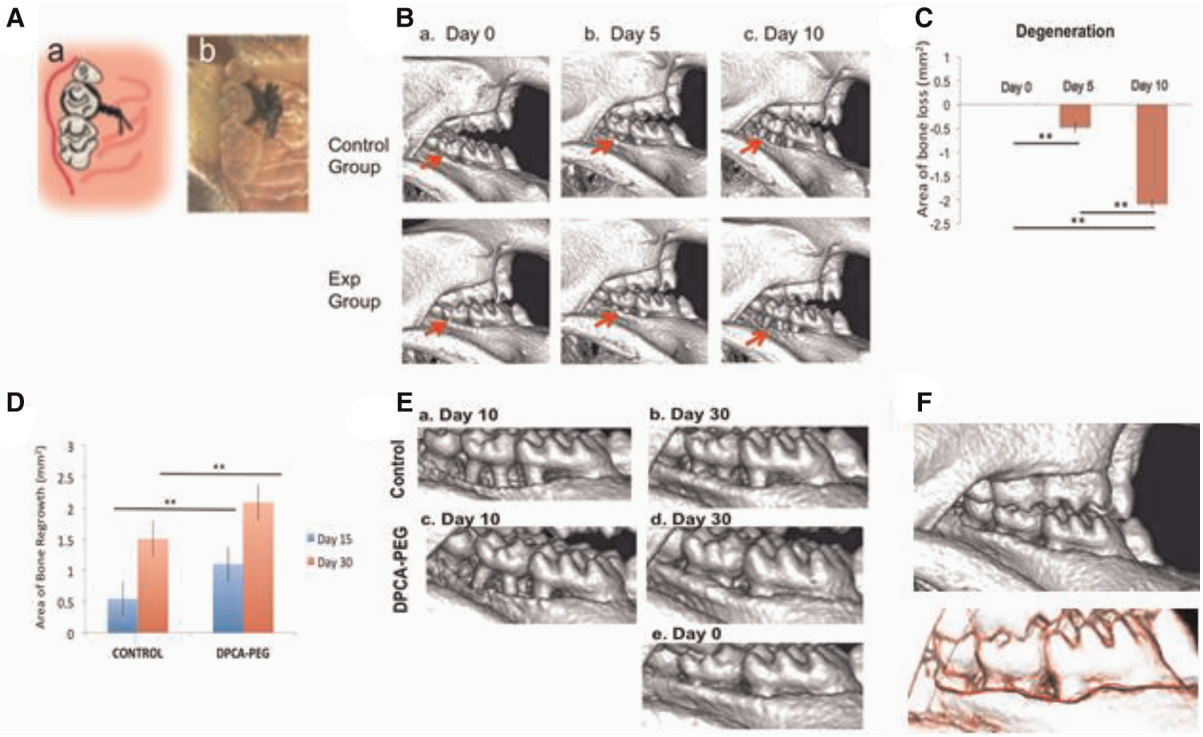



FIGURE 1
Degeneration of the mouse jawbone in the presence of ligature followed by regeneration of the jawbone post-ligature and post-drug. (**Aab**) The ligature-induced periodontitis model. 5–0 silk suture was passed through the interdentium between the maxillary first molar, the second molar and third molar using Dumont forceps. Suture was tied firmly using a triple-knot and excess suture was cut using spring scissors as seen in the cartoon (**a**) and photomicrograph (**b**). Taken from Ref. (31). (**Ba–c**) Micro-CT scans of jaws from mice during the 10 day ligature period for days 0, 5, and 10. The control group image is representative of the group ($n = 3$) that will not receive drug (control, upper row) and the experimental group image is representative of the group ($n = 3$) that will receive drug (experimental, lower row). The red arrows show the maxillary left second molars with bone degeneration extending to the adjacent 1st and 3rd molars. For visual clarity, the images are inverted 180 degrees (now: mandible top, maxilla bottom). (**C**) The area of degeneration was determined for all animals tested and shown for day 5 and day 10 after ligature placement. The *y*-axis is: Area of bone loss ($mm^2$); ($n = 7$); error bars represent standard errors; for days 0–5, $p = 0.00289$; for days 0–10, $p = 7.33084 \times 10^{-11}$; and for days 5–10, $p = 3.60818 \times 10^{-7}$. (**) Represents $p < 0.01$. In (**D**), a graph of area of bone growth is seen for mice post–ligature but not given drug (no drug control) vs. mice given drug (DPCA-PEG). Mice were injected with DPCA-PEG subcutaneously on days 0 and 8 after ligature placement and removal. MicroCT scans were obtained on day 15 of the experiment (day 5 post-ligature) and on day 30 (day 20 post-ligature). Here, a statistical analysis of the area of bone growth ($mm^2$) is seen. Significant differences are found between the no drug control mice ($n = 10$) and DPCA-PEG-treated mice ($p = 0.00253$) on day 15 (blue bars) ($n = 12$). The same is true on day 30, where DPCA-PEG-treated mice showed highly significant differences from non-drug-treated controls ($p = 0.00612$) (red bars). Area analysis was performed as described in the Materials and Methods. The *Y*-axis = Area of bone regrowth ($mm^2$); the error bars represent standard errors; and *p* values are represented as (*) = $p < 0.05$; (**) = $p < 0.01$. Mouse jaws analyzed were $n = 10$ for ligature, no drug; $n = 12$ for ligature/plus drug. In (**E**) micro-CT data shows a representative mouse maxilla which had ligature removed at day 10, scanned on day 10 and then re-scanned on day 30 (**Ea,b**) as compared to a representative mouse maxilla receiving ligature and DPCA-PEG drug seen on day 10 and day 30 (**Ec,d**). The level of regrowth in the drug-treated group shows an almost, if not complete, return to what is seen before the start of the experiment (da0) (**Ed,e**). In (**F**), there was no change in bone histology approximately 6 months later. Mice injected with DPCA-PEG drug were kept for additional observation as they aged. Over six months after the da 30 scan, mice were rescanned (upper panel) and then compared to the day 30 scan by overlaying the two scans on da 30 and da 220 (lower panel). The black line is the da 30 scan and the red line is the da 220 scan. Though shown as two lines, they are exactly overlapping. This result is representative of three mice.

analysis showed significant bone degeneration within 5 days continuing to day 10 (**Figure 1Ba–c**). The ligature was well tolerated with no obvious ill effects such as reduced chow ingestion. Graphed values of bone loss are seen in **Figure 1C**.

## Changes in bone regrowth in mice given DPCA-containing drug

At day 10, ligature was removed and mice were separated into two groups: 1) the control group treated no further; and 2) a group injected twice (days 10 and 18) with DPCA-PEG (29). Mice were then Micro-CT-imaged on days 15, 21, and 30 (**Supplementary Figure S1**).

Three separate experiments were carried out, and Micro-CT results quantified. The key measure of alveolar bone loss and subsequent bone regeneration is derived by calculating changes in bone area assessed from lateral images using the cementum-enamel junction (CEJ) and crest of the buccal alveolar bone as anatomic landmarks (**Figures 1D,E** and **Supplementary Figure S1**) to determine changes in each jaw. Statistical analyses were performed and statistically significant differences in bone area of treated and control groups were seen. These differences demonstrate the efficacy of DPCA-PEG in reversing induced periodontal disease in this mouse model. In **Supplementary Figure S1**, jaws are also shown digitally overlaid and aligned on top of one another to show changes in bone growth. In **Figure 1D**, treatments and degree of increase/decrease in area in $mm^2$ is seen with highly significant changes after drug treatment.

Thus, DPCA-PEG induced rapid and significant bone regrowth. Comparing jaw from the day ligature is removed where extreme jawbone degeneration is seen by day 10 (**Figure 1Ea,c**), one can see dramatic bone re-growth (**Figures 1D,Ed**, Day 20 after ligature removal). Regrowth of alveolar bone in mice given DPCA-PEG is nearly, if not completely, recovered compared to images taken before ligature and drug treatment, day 0 (**Figure 1Ee**). Not only does bone length and apparent bulk volume return to normal within 30 days, but morphology of regenerated bone is indistinguishable from normal alveolar bone. Thus, the thickened boney alveolar ridge adjacent to the crowns is fully restored in drug-treated mice.

It should also be noted that control bone itself does grow back, albeit to a significantly less extent, consistent with earlier observations (43, 44). This is not surprising since it has been long established that many rodent species continually wear down tooth crowns with subsequent regrowth of tooth roots and surrounding alveolar bone (see Discussion).

Finally, three ligature plus DPCA-PEG-injected mice were examined for any long-term effects or reversal of healed injury. After approximately six months, bone morphology was still stable. As seen in **Figure 1F**, no reversal of healing nor adverse effects of drug on the health of the mice were seen.

## Soft tissue changes post-ligature and post-drug

Bone loss in PD is preceded by bacterially-induced inflammatory changes in soft tissue (gingiva) characterized often by swelling and bleeding on probing. As bone loss continues, the inflammatory state is intensified with breakdown of attachment fibers between supporting alveolar bone and roots of the teeth. The combination of direct bone loss and breakdown of periodontal (PDL) fibers leads to tooth mobility and eventual tooth loss. It was thus important to observe whether drug-induced regenerative therapy restored not only bone, but also soft tissue integrity.

In **Figures 2Aa–c,Da**, normal tissue histology around the tooth is shown with rete pegs in the epidermis, un-inflamed dermis, rich cellular pulp, and a PDL surrounding the full tooth root.

In **Figure 2Ba–c**, tissue from ligature-only-treated mice is seen on day 30. There is ablation of PDL root-bone attachment down to the root apex (green arrows). Furthermore, there is increased porosity of the surrounding alveolar bone (**Figure 2Db**). The human clinical correlate might be extreme PD with tooth extraction indicated. Compared to some alveolar bone recovery seen by micro-CT (**Figure 1Ea,b**), soft tissue recovery is poor by day 30 (**Figures 2Ba,c,E**).

**Figure 2Ca–c** shows tissue after ligature plus drug (day 30). Cell-rich reattached PDL fully surrounding the root (**Figures 2Cc,E**), alveolar bone with less porosity (**Figure 2Dc**), and a highly vascularized pulp (**Figure 2Cb**) is found. PDL fibers have returned (**Figures 2Cc,Dc**).

## Vascular changes in pulp and increases in stem cell markers post drug

Twenty days after drug initiation (day 30), there was an almost 8-fold increase in cellularity and vascularity in B6 pulp after drug vs. no drug control (**Figures 2Cb**, **3A**). Neurofilament IHC, used to measure nerve in highly innervated pulp showed increases in B6 mouse jaws after ligature plus drug (**Figure 3E**). Increases in early stem cell markers such as OCT3/4 and PAX7 (**Figures 3C,D**) and the mesenchymal stem cell marker aSMA (**Figure 3F**) were also seen. Two markers, SOX2 and CD34 showed no differences with or without drug.

## Changes in PDL markers

A closer examination of B6 PDL showed that after ligature plus drug, the number of PDL fibroblasts increased two-fold compared to ligature alone. This was carried out examining longitudinal cross-sections (**Figure 4A**) and transverse cross-sections (**Supplementary Figure S2**), both giving the same result.

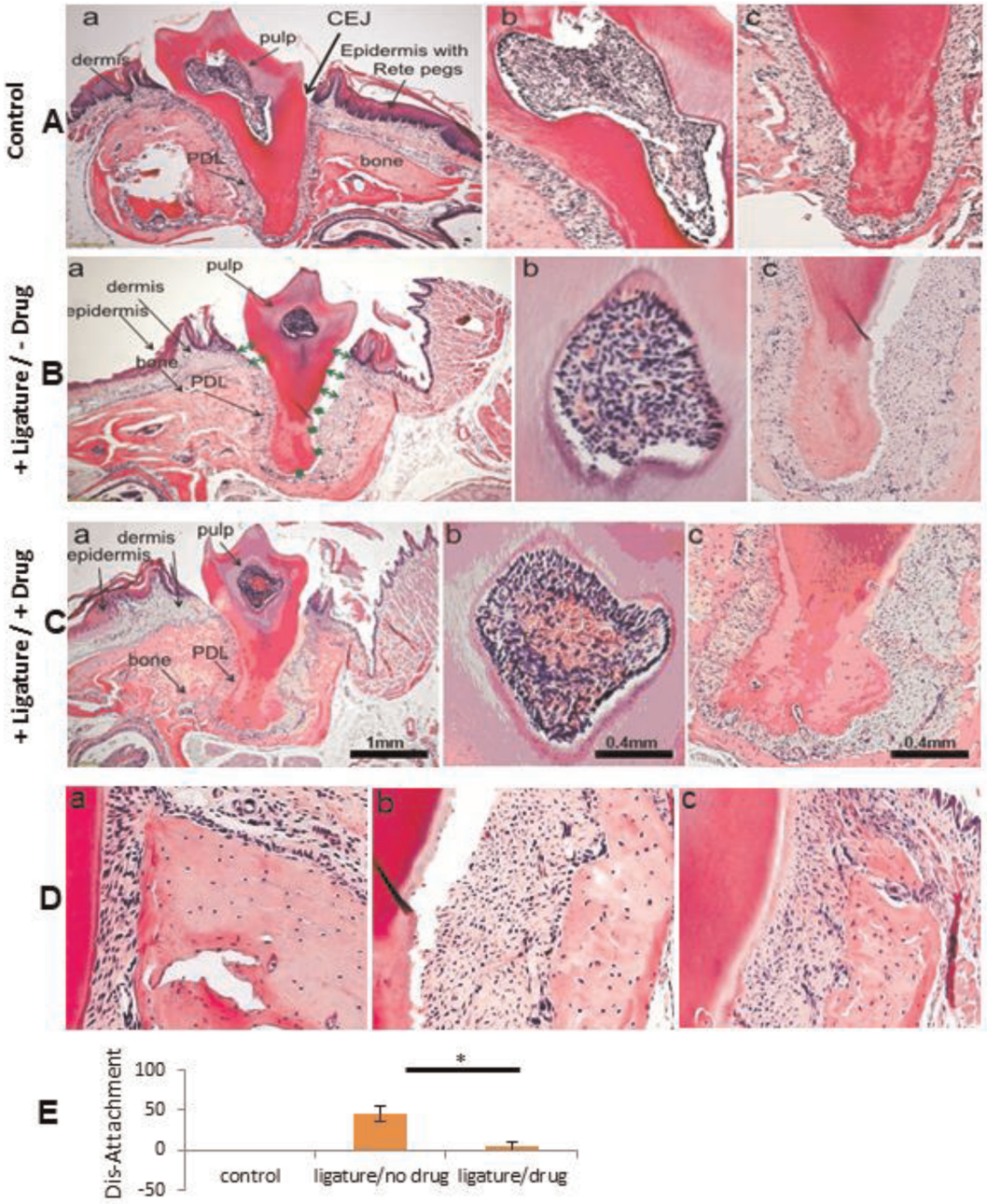



FIGURE 2
Histological analysis of normal and 20 days post-ligature +/- drug treated jaw tissue. In figure, H&E-stained lateral jaw sections of a maxillary second molar are seen. The scale bar = 1mm for (**A-Ca**). The scale bar = 0.4 mm for all other photomicrographs, marked and unmarked. In row A, a normal mouse maxilla is seen (**Aa-c**). In row B, a maxilla from a +ligature/-drug-treated mouse is seen (**Ba-c**); and in row C, a maxilla from a +ligature/+DPCA-PEG-treated mouse (**Ca-c**) is seen on experimental day 30 (day 20 post ligature) and all samples are representative of 3 different mice for each condition. (**a's**) show tooth and surrounding tissue; (**b's**) show pulp; (**c's**) show PDL. (**D**) shows a high magnification of bone from a normal maxilla (**Da**), from a maxilla of a +ligature/-drug-treated mouse (**Db**), and from a maxilla of a +ligature/+DPCA-PEG-treated mouse (**Dc**). In the normal control mouse (**Aa**) is seen a thick gingival dermis and epidermis with Rete pegs, a rich periodontal ligament attachment extending from root tip to cemento-enamel junction (CEJ), with periodontal ligament fibers and fibroblasts attached from tooth to normal bone (**Da**), along with pulp chamber and its rich cellular composition. The pathology induced from a silk ligature around the root adjacent to the crown is seen in (**Aab**). (**B,C**). In the +ligature-treated/-drug-treated mouse (**Bac**), a space between PDL and tooth extending to both sides of the tooth apex (green arrows, stars) is seen. Breakdown of dermis and epidermis surrounding the tooth crown with obvious dis-attachment of PDL to root surface results in a deep invagination extending > 60% of the length of the CEJ to tooth APEX. PDL is totally eliminated on the right of (**Bac**) and partially obliterated on the left side. Epidermis and dermis are tattered and edematous. Bone shows porous changes (**Db**) and clinically this tooth would be highly mobile within the tooth socket and correspond in humans to advanced PD involvement within an indication for extraction. The jaw in (**Ca**) is from a +ligature/+drug-treated (2 doses DPCA-PEG, da0, da8 post-ligature) mouse. Here, PDL is attached to bone (**Dc**) and tooth, dermis is richer than without drug-treatment. Pulp shows higher levels of blood cells and vessels. A higher magnification of pulp shows differences with different treatments (**Ab,Bb,Cb**). After drug treatment, the pulp is richer with higher levels of red cells and angiogenesis compared to both normal and ligature alone-treated mice (pink/red).

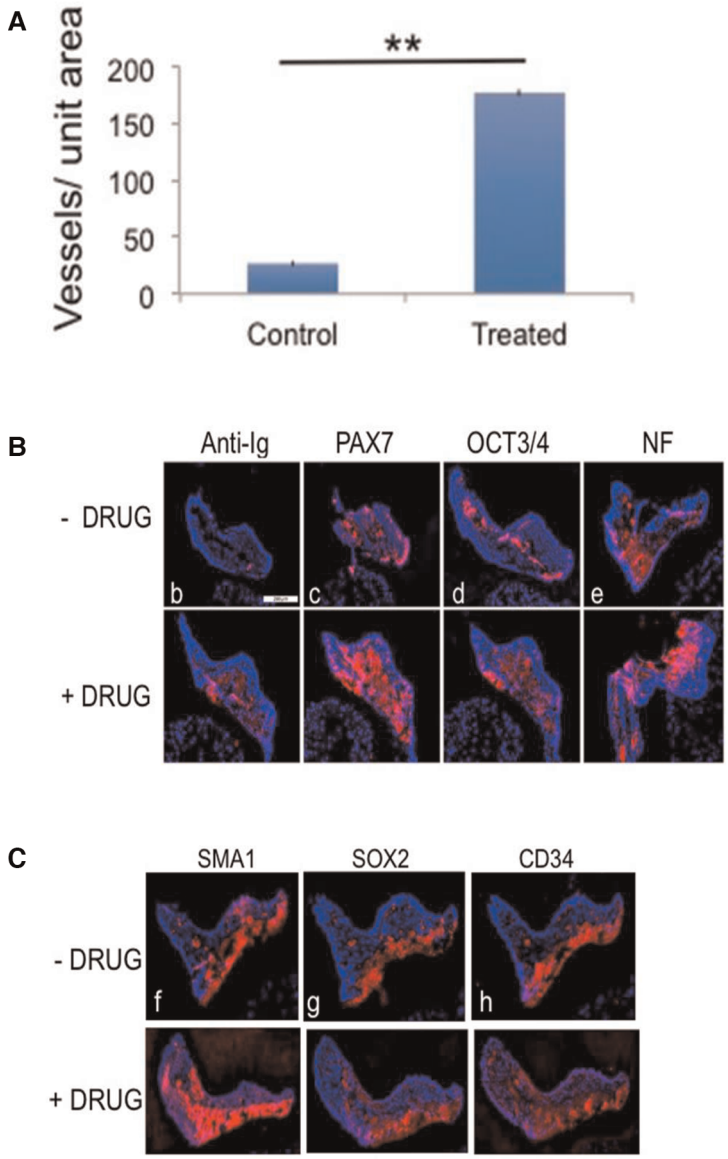



FIGURE 3
Stem cell marker expression in pulp after ligature and drug-treatment. In (A), a graph of the level of angiogenesis in the pulp on day 30 (Figures 2Bb–Cb) is presented. The level of red in the pulp was determined by determining the number of pixels of red (IHC staining) using Photoshop as compared to the number of pulp area pixels (blue, DAPI) and is shown as a graph of the level of red in (A). The *Y*-axis is the vessel area/pulp area in the control and drug-treated mice; $n = 4$; the error bars are standard errors; the $p = 0.0027127$ and (**) represents $p < 0.01$. Stem cell or de-differentiation marker expression for PAX7, OCT3/4, aSMA (SMA1), SOX2, and CD34 was determined by IHC (red) in the pulp and is seen in (B–H). The anti-Ig control is lower in drug-untreated than treated samples. The scale bar = 200 um for all photomicrographs. The pulp was also examined for expression levels of neurofilament (NF), an intermediate nerve fiber filament and marker of innervation (E). Quantitative staining using Photoshop CS6 determined the # red pixels over the total number of pixels in the pulp giving % positive staining; (n = 4) and percentage of red pixels to total pixels determined. For (B–H), the no drug control vs. plus drug experimental is seen for (B) (0.7% vs. 6%); (C) (14% vs. 38%); (D) (9% vs. 21%); (E) (17.5% vs. 27%); (F) (19% vs. 38%); (G) (12.5% vs. 13%); and (H) (18% vs. 18%). Two markers, SOX2 and CD34, did not show a difference with and without drug on day 30.

Given that 1,4-DPCA blocks PHDs and leads to HIF-1α stabilization, we noted high HIF-1α expression levels in PDL (Figure 4B). High expression levels of scleraxis, a marker of activated PDL (Figures 4C,C′), was observed post ligature plus drug in the pulp chamber and canal. In previous studies (23, 29), after DPCA-PEG treatment of ear pinna wounds and a regenerative response, we noted expression of de-differentiation and immature cell markers. Here, we saw in both PDL and pulp including the apical pulp canal, all of which contain stem cell progenitors, an increased immunostaining for stem cell markers PAX7 and OCT3/4 (Figures 4E,F), and MSC markers aSMA, SOX2, and CD34

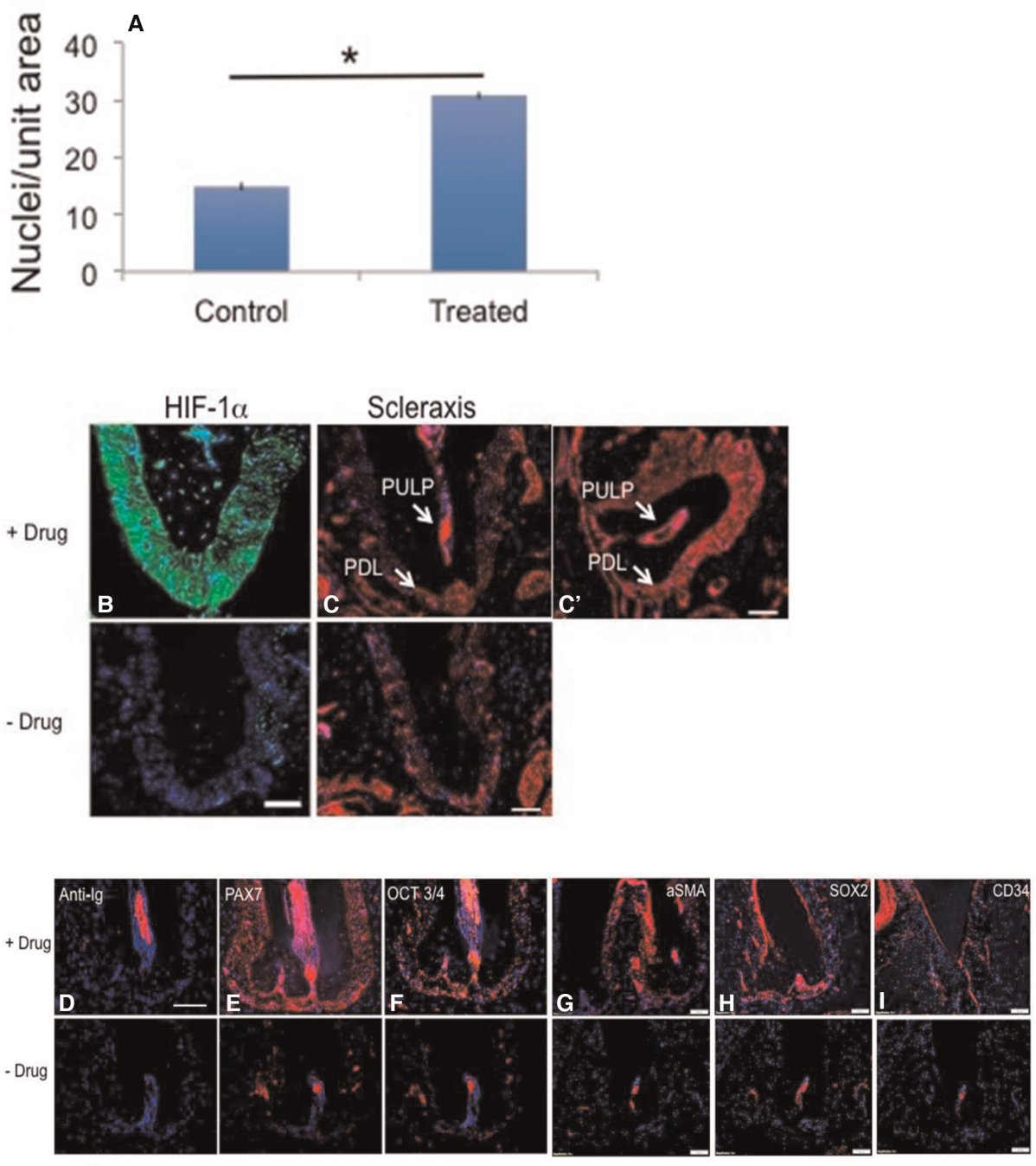



FIGURE 4
Stem cell marker expression in PDL after ligature and drug-treatment. In figure, PDL analysis was carried out in B6-treated mice. In (**A**), in B6 mice, the number of PDL fibroblast nuclei were counted from Figures 2Bc,Cc and are two-fold greater in the drug-treated B6 mice. The *Y*-axis is the number of nuclei/unit area; error bars are standard errors; $n = 4$; $*p < 0.01$. In (**B**,**C**,**C'**), longitudinal jaw cross-sections from ligated-plus-drug (upper panels) vs. ligated only (lower panels) respectively were stained with antibody to HIF-1α (green) and scleraxis (red) with PDL staining levels higher in sections from drug-treated vs. no drug mice (for scleraxis, 11% vs. 4% red pixels; as described in Figure 3) (**C**). The scale bar = 100 um. In (**E**–**I**), antibodies to the stem cell or de-differentiation markers PAX7, OCT3/4, aSMA, SOX2, and CD34 stained more highly in the drug-treated PDL (upper panels) compared to the non-drug treated PDL (lower panels). Anti-Ig controls showed no staining in the PDL except in the root canal (**D**). The scale bar = 100 um for all photomicrographs except Fig 4d where the scale bar = 200 um.

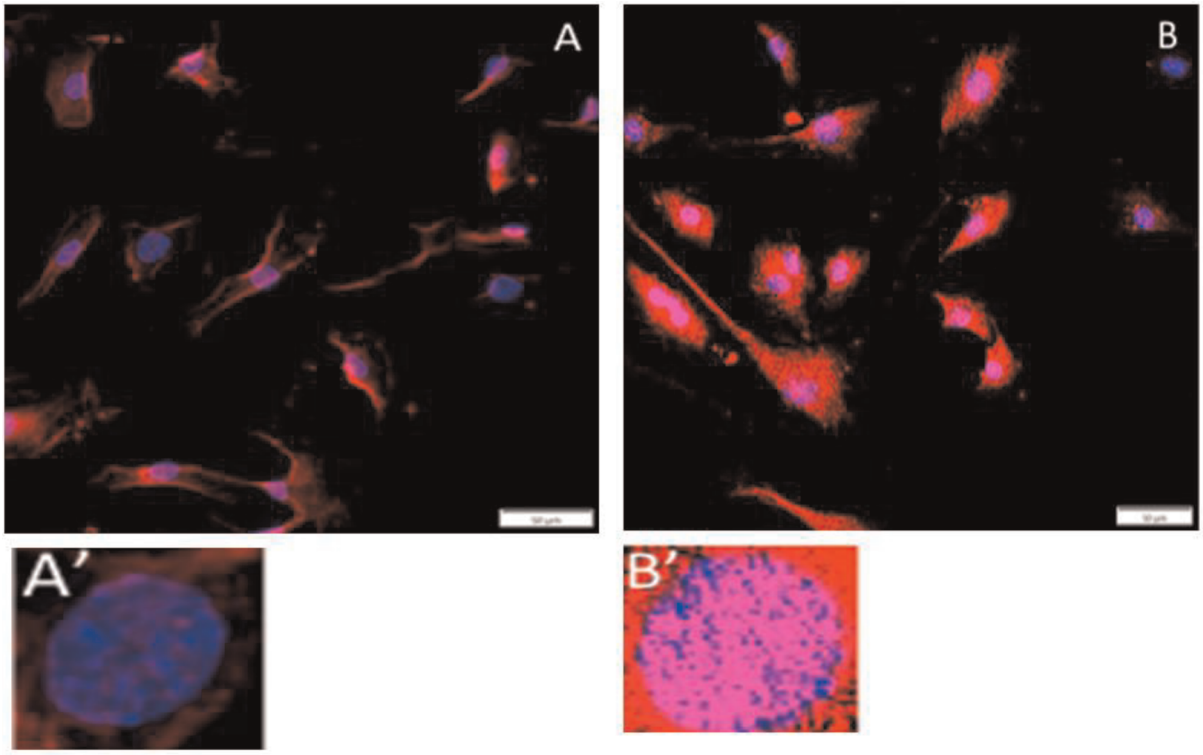



FIGURE 5
In-vitro nuclear expression of stem cell or de-differentiation markers. In figure, gingival fibroblast-like cells from normal B6 female mice were grown on coverslips, treated without (A,A') or with 1,4-DPCA (100 uM) (B,B') overnight in culture, and stained with antibody to OCT3/4 (red) and the nuclei stained with DAPI (blue). OCT3/4 staining is seen in the cytoplasm in DPCA-untreated cells and seen increased in the cytoplasm but now also found in the nucleus in DPCA-treated cells. The scale bars = 50um.

(Figures 4G–I). Interestingly, SOX2 and CD34 showed no changes in the pulp (Figures 3G,H).

The cellular location of stem cell markers (cytoplasm vs. nucleus) could not be determined using tissue sections. Therefore, in-vitro analysis of stem cell marker OCT ¾ location in cell culture was examined pre and post addition of 1,4-DPCA (Figure 5). Using gingival cells in the absence of DPCA (Figures 5A,A′), we found OCT3/4 expression in the cytoplasm but not nucleus. After treatment with DPCA (100 uM) in-vitro, all cells showed both nuclear and cytoplasmic localization of OCT3/4 (Figures 5B,B′). The amount of OCT3/4 staining increased after DPCA treatment.

## Metabolic reprogramming

Treatment of mice with DPCA-PEG also led to gene expression changes in jaw tissue in molecules related to the aerobic glycolysis metabolic state (Figure 6). *Gapdh, Glut1, Pdk1, Pgk1, and Ldha* showed increased mRNA expression levels in ligature + plus drug-treated mouse jaw tissue (da 30) obtained from isolated paraffin-embedded tissue sections as seen in Figure 2 containing bone, tooth, gingiva, and PDL. All genes showed the same expression patterns with high levels after ligature plus drug but low expression levels with ligature or drug alone.

# Discussion

## Induced periodontal disease as a model for bone and soft tissue regeneration

Experimental bone injuries *in vivo* generally require surgical incisions through overlaying skin, fascia and muscle. Periodontal disease, however, arises from a bacterial overgrowth of resident intra-oral micro-organisms external to soft tissues (the gums) of the oral cavity with subsequent host inflammatory responses leading to massive lesions of underlying alveolar bone. An induced periodontal disease model is therefore attractive from the perspectives of both basic bone regeneration biology and as a translational stepping stone in the treatment of a common human disease.

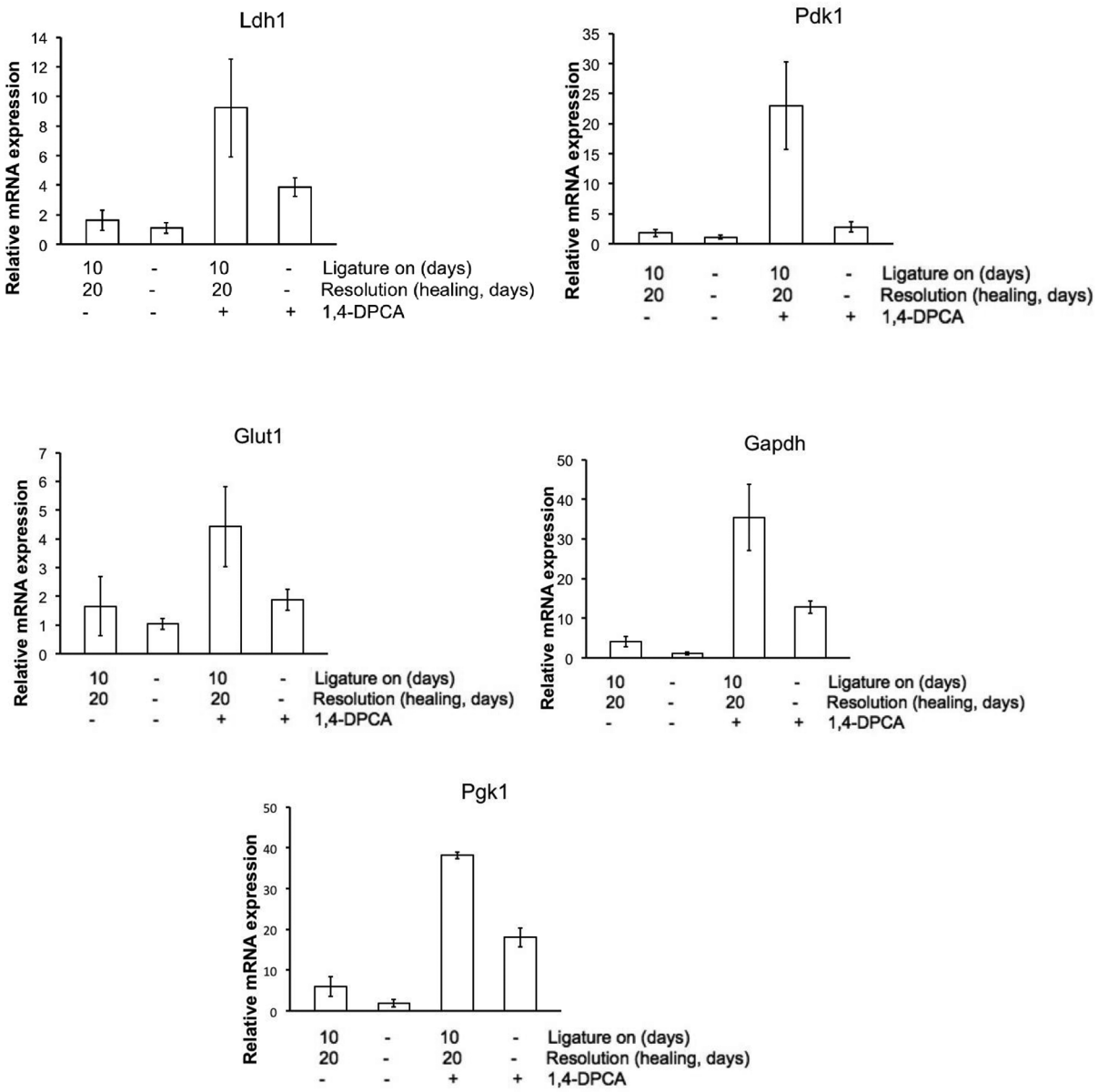



FIGURE 6
Metabolic gene expression in jaw tissue. In figure, mRNA was extracted from 2 sets of jaw tissue as seen in Figure 2. QPCR was carried out and results show that for all metabolic genes associated with aerobic glycolysis, high expression levels were seen in tissue from mice treated with both 1,4-DPCA and with ligature placed round the 2nd molar. These results were done in triplicate ($n = 3$). The $p$-value differences between ligature with DPCA (3rd bar) and without DPCA (1st bar) were for LDHa, $p = 0.0439$*; Pdk1, $p = 0.0095$**; Glut1, $p = 0.0922$ !; Gapdh, $p = 0.0103$*; and Pgk1, $p = 0.0001$**. These values were (a) highly suggestive (!), (b) significant (*), and (c) highly significant (**).

With declining incidence of caries, periodontal disease (PD) has emerged as the most common cause of tooth loss affecting 30%–60% of the adult population, presenting a major clinical challenge (45, 46) with the loss of supporting alveolar bone around the roots of teeth as well as the destruction of adjacent soft tissues (47) and can involve a single tooth or the entire dentition. PD etiology is due to a number of identified periodontopathic organisms found in the gingival crevice (31–33, 47) combined with host susceptibility factors such as diabetes (48, 49).

The challenge in advanced PD, where bone loss may exceed 50%–80% of the length of the tooth root, is to halt further progression and achieve a functionally and esthetically acceptable outcome. Thus, a primary goal is to restore lost alveolar bone and soft tissues to the original state without loss of teeth (50, 51). It is for this reason that we have explored

PHDi-based regenerative drug-induced bone and soft tissue regeneration in a PD model.

## The role of HIF-1α and changes in vascularization

The biphasic high expression of HIF-1α and its target genes was shown to be a critical molecular tissue regeneration pathway as determined from previous soft-tissue studies (16, 23, 29). These target genes include *Pdk*, and *Ldha, Pkm2*, and *Gapdh*, and *Pgk1*, all glycolytic enzymes (**Figure 6**) which lead to metabolic remodeling or reprogramming (21, 22, 24–27). These molecules required two signals for elevated expression, drug + injury (ligature). HIF-1*α* was identified as the molecule responsible for the broad regenerative ability of the MRL mouse from multiple directions. First, in genetic mapping studies of MRL mice, a reduced level of the gene RNF7, part of the ubiquitin ligase complex, necessary for proteolysis and lowered protein expression levels of HIF-1α (52), proved to be a candidate gene associated with regenerative healing (53). Furthermore, MRL mice were found to be metabolically more embryonic using aerobic glycolysis regulated by HIF-1α target genes (19, 20). Finally, *siHIF1a* completely suppressed ear hole closure both in the MRL and non-regenerative mice treated with 1,4-DPCA-hydrogel (23).

One of the many intriguing aspects of HIF-1α elevation is the de-differentiation of mature cell populations to an immature state determined by changes in molecular markers such as Nanog and Oct ¾ regulated by HIF-1α and aerobic glycolysis (54–61). This state characterizes cells in the amphibian regenerative blastema and is thought to be key to the regenerative response (62). Also, HIF-1α is responsible for an enhanced vascularization response, producing molecules such as VEGF and HMOX1. It is clear in the tooth pulp that vascular tissue is significantly increased 15–20 days after DPCA-PEG administration (**Figures 2Cb**, **3A**). This change in vascularity could lead to an increased number of stem cells migrating into the tooth as noted by stem cell marker increase (**Figures 3B–F**). On the other hand, DPCA-PEG could lead to de-differentiation with increased stem cell markers and growth as seen in the ear accompanying regenerative ear hole closure (23, 29).

## Induction of stem cell markers

Many molecular and cellular markers of regeneration observed in spontaneously regenerating species such as the newt and axolotl (1–3) and in the MRL mouse (19) were indistinguishable from those seen in DPCA-PEG-treated mice (23, 29, 16). Our previous studies largely focused on soft tissue, specifically in the ear pinna and included regrowth of hair follicles and cartilage (23, 29). In the PD model studied here, not only soft tissue but also bone is affected by the drug. Multiple early impacts (5 days post-ligature) using a different form of the drug, DPCA-hydrogel, included increased Treg FOXP3 + populations and lowered inflammatory cytokines (63). In the current study using DPCA-PEG, between 20 days and as long as 220 days post -ligature, the PDL, which is key to supporting the teeth and securing them to the bone, showed complete re-attachment to the teeth. Increased numbers of PDL fibroblasts in the drug group are also seen. PDL also showed increased levels of scleraxis, a transcription factor considered to be a PDL marker (34–37) associated with osteocytes and cementoblasts (36).

Another dental soft tissue target, the pulp, is considered to be a source of stem cells and contains dentinoblasts which re-line the inside of the tooth dentin. We noted after drug that the pulp vasculature as well as stem cell markers were increased. As mentioned above, whether this is due to a de-differentiation process in the pulp or to increased numbers of stem cells in the over-abundant vasculature is not clear. We also noted an increase of neurofilament (38), a nerve marker, which is present in normal pulp and increased after drug.

Stem cell populations have been identified that are associated with teeth in the pulp chamber, at the base of growing roots, and in the PDL (40). Progenitor cell populations have been identified previously in periodontal tissue and express mesenchymal stem cell markers such as STRO-1, CD146, CD44, and αSMA (30–33, 64–66). These progenitors exhibit many stem-cell-like features, including small size, responsiveness to stimulating factors, slow cycle time and the ability to generate multiple mesenchymal lineages (67–69). In addition, neural crest-derived cells have been identified in the periodontal ligament and the pulp chamber using markers such as Slug, AP2 alpha, HNK-1, p75NTR and Nestin (70–74).

Alpha SMA has been found in stem cells and regenerating tissue and in blood vessel pericytes and myoepithelial cells involving force-generating function. During mandible development, aSMA was found in the dental follicle, in the periostin-positive area along with RUNX2 positive cells, and localizes to the alveolar bone region suggesting involvement in bone formation (75).

We tested multiple stem cell markers such as OCT3/4, PAX7, SOX2, CD34 and aSMA in the jaw. These de-differentiation markers were found in pulp and PDL and increased after DPCA-PEG. Again, these markers could be due to the dedifferentiation of mature cells (1–3, 23, 30, 54–61) or could be markers of stem cell populations in the pulp and PDL as discussed above (76). Cross talk between the pulp and periodontal ligament should not be ruled out since pulp and PDL are anatomically connected *via* the apical root vasculature.

Although stem cell markers were increased in PDL with DPCA- PEG, it was difficult to see cellular sub-structure and intra-cellular location of these stem cell markers. We grew gingival-derived fibroblast-like cells to analyze the staining of OCT ¾ with and without addition of DPCA. Here, we found that cells before DPCA showed staining in the cytoplasm, but after DPCA, staining was seen in the nucleus where Oct ¾ acts as a transcription factor and is key to self-renewal of undifferentiated embryonic stem cells and is a specific marker for dedifferentiation (51).

Taken together, our present work has shown the ability of systemic DPCA-PEG administration to regenerate severely degraded alveolar bone and soft tissue with remarkable anatomic fidelity within 20 days post drug.

It should be noted that in humans, regenerative recovery of bone is seen, but is usually limited to bone fractures which are aligned with a gap of less than 1 mm (77, 78). On the other hand, it is also known that both the maxillary and mandibular incisor teeth of rodents kept in animal colonies on soft chow often elongate (33, 34) and we are seeing a background growth effect. In studies reported here however, the regenerative contribution of the drug is clearly distinguishable from background.

In conclusion, this study extends our previous work on drug-mediated stabilization of HIF-1α to achieve soft tissue regeneration in mice (16, 23, 29, 59) in new directions. First, systemic DPCA-PEG rapidly reverses severe bone loss in an anatomically complex structure, alveolar bone of the maxilla, leading to a nearly perfect replica of healthy bone and associated soft tissue such as PDL with long-term maintenance. This induced bone loss occurs in an experimental system which emulates the bacterial etiology of the human disease, periodontitis.

# Materials and methods

## Study design

We used the inbred mouse strain, C57BL/6 female, to study the effect of a small-molecule inhibitor of PHDs on the in-vivo expression of HIF-1α and the impact on quantitative regenerative maxillary bone growth (63) and soft tissue regeneration including PDL and pulp. A 5.0 silk ligature model was used to induce periodontitis in the mice and DPCA–PEG was tested and subcutaneously implanted in the back of the neck at multiple time points (**Figure 7**). End points of the study were previously determined to be a minimal of 30 days and up to 220 days after injury and included key indices of tissue regeneration such as bone regrowth as measured by Micro-CT scanning, soft tissue regrowth after H&E histological analysis including epidermis, gingiva, PDL attachment and cell number, and markers of regeneration determined by immunohistochemistry of jaw tissue for stem cell markers, and HIF-1α, neurofilament, and scleraxis and RT-PCR analysis of gingiva. These parameters involved physical measurements of growth and gene expression at the RNA and protein levels. Tissue was coded and different laboratory personnel were involved in doing ligatures, scanning, tissue preparation, and data analysis.

## Animals

C57BL/6 female mice, 9 weeks of age, were obtained from Taconic Laboratories. The experiment was done with 2 groups of 3–4 mice based on the previous work of Hajishengallis

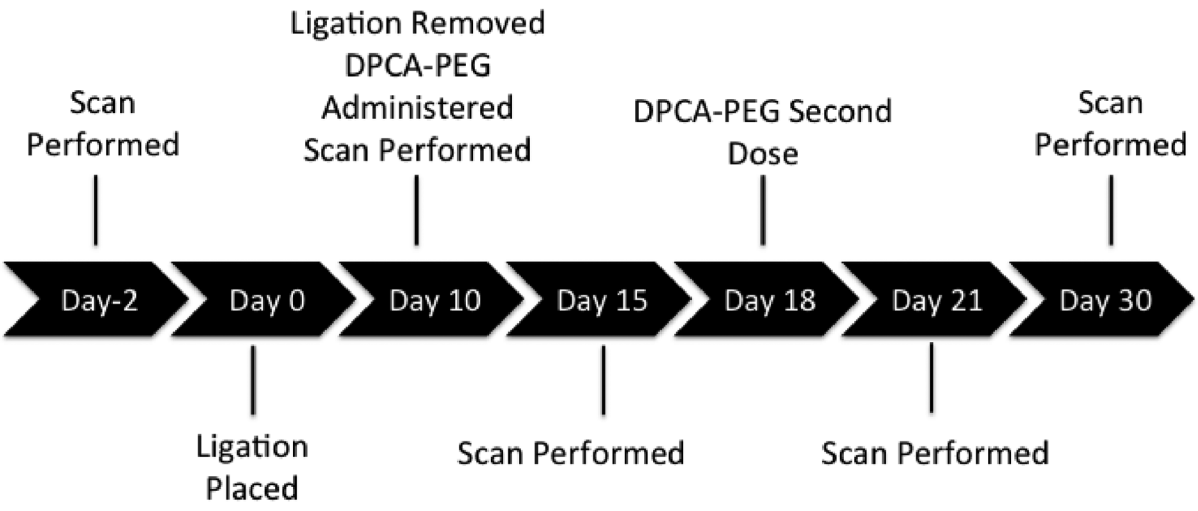



FIGURE 7
Timeline of treatments.

et al. (31, 63) and was repeated twice for a total of 10 mice in each group.

Food and water were provided *ad libitum*. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of LIMR and were performed in compliance with institutional, state, and federal policies.

## Tissue culture

Primary fibroblast-like cell lines from gingival tissue were established from B6 female mice by plating in dispase and then collagenase and then grown in DMEM-10% FBS supplemented with 2 mM L-glutamine, 100 IU/ml penicillin streptomycin and maintained at 37 °C, 5% CO2, and 21% O2. The cells were washed and only adherent fibroblasts maintained. Cells were split 1 : 5 as needed to maintain exponential growth and avoid contact inhibition. Passage numbers were documented and cells from early passages (<P20) frozen in liquid nitrogen and used in the described experiments.

For immunohistochemical staining, primary gingival fibroblasts were grown on coverslips in DMEM with 10% FBS at 37 °C in a humidified 5% CO2 incubator. Before staining, cells were incubated with DPCA overnight. The coverslips were rinsed with 1× PBS, the cells were fixed in cold methanol (−20 °C) for 10 min, rinsed with 1× PBS, treated with 0.1% Triton-X100, and then incubated with the appropriate primary and secondary antibodies (**Supplementary Table S1**). Photomicrographs were produced using the fluorescent microscope (Olympus AX70) and a DP74 camera with CellSens Standard software for image analysis.

## Drug application

DPCA-PEG was synthesized as described (29) and was injected subcutaneously on day 0 and day 8 using 25 ul of gel #10/injection or 50 ug of DPCA. The time course of injections and scans performed during longitudinal experiments can be seen in **Figure 7**.

## Preparation and immunohistochemistry of jaw tissue

Tissue from upper jaws was fixed in Prefer fixative (the active ingredient is glyoxal) (Anatech) for 5 days and then washed in $H_20$. Jaw tissue was then decalcified using UltraPure 0.5 M EDTA, pH 8.0 for 5 weeks with changes in EDTA solution twice a week. Tissue was put in ETOH and then embedded in paraffin and 5-$\mu$m thick sections cut. Before staining, slides were dewaxed in xylene and rehydrated. Tissue sections were then treated with 3% $H_2O_2$ and nonspecific binding was blocked with 4% BSA (A7906; Sigma) for 1 h. Primary antibodies and matched secondary antibodies used for IHC are shown in **Supplementary Table S1**. Photomicrographs were produced using the fluorescent microscope (Olympus AX70) with an Olympus D74 Color Camera using CellSens imaging software.

For histological stains, tissue sections were treated as above and stained with Hematoxylin (Leica Microsystems, #3801562) and Eosin (Leica Microsystems, #3801602). The slides were washed, rehydrated, cleared with Xylene and coverslipped with Permount mounting media (Fisher, SP15-500). Staining was visualized using an Olympus (AX70) microscope in bright field for H&E and fluorescence.

## Induction of periodontal disease

For ligature placement, mice were anesthetized with Ketamine/Xylazine, a 5–0 silk ligature was placed around the upper left second molar of the maxilla according to an established procedure (31) generating a dental plaque-retentive milieu that reliably and quantitatively produces a periodontal bone lesion. Importantly, this ligature procedure induces not only bacterial over-growth but also selective expansion of periodontopathic microorganisms, mimicking quantitative and qualitative (dysbiotic) microbiome alterations, the same etiology as that in human disease (32, 33). Upon placement of the ligature, the 5–0 silk ligation accumulates dental plaque and oral bacteria creating a local inflammatory state in the surrounding gums and bone of the tooth. Ketamine/Xylazine was used to remove the ligature and mice were re-scanned. Mice were subsequently scanned for further analysis. During this time, mice were kept on a normal diet of mouse chow and were monitored daily for any signs of physical discomfort in accordance with the Lankenau Institute for Medical Research (LIMR) Animal Care Policies and Procedures Manual. At each time point, animals were imaged and analyzed with the area analysis described below. On four occasions the ligature model was not completed due to the 5–0 silk ligation falling off of the tooth. These mice were excluded from the study and the remaining mice were randomly distributed before any DPCA-PEG injections.

## Ketamine/xylazine mixture

Stock solutions of Ketamine (100 mg/ml, Hanna) and Xylazine (20 mg/ml, Hanna) are prepared using PBS as a diluent, respectively at a ratio of 3:1:16, and is vortexed and used immediately.

## Micro-CT scanning

For scanning, mice were anesthetized using isoflurane (Henry Schein) 2%–4% concentration in 100% $O_2$ for 3 min in an anesthesia chamber. Upon establishing anesthesia, mice were placed into the microCT FX (Perkin Elmer; ref 80) tray with the isoflurane nose cone. 3D images were collected and rendered with a voltage of 90 kV, a CT current of 160 $\mu$A and a live current of 80 µA for 17 s for a total dose of 11milliGY (79, 80). This constitutes the pre-intervention baseline.

Buccal images were analyzed unlike original experiments done by measuring the palatal side without Micro-CT (31, 63). They were normalized to the Micro-CT HA D4.5 phantom from QRM (Quality Assurance in Radiology and Medicine GmbH).

## Micro-CT analysis

An analytical method to quantitate longitudinal changes in bone morphology was designed and implemented. 3D renderings were obtained using Quantum software (Perkin Elmer) and subsequently converted to 2D images, which were then superimposed in Photoshop (CC 2019) for quantitation of bone morphological changes.

## RNA isolation and quantitative qPCR

Total RNA isolation from paraffin-embedded jaw samples was performed using RNeasy DSP FFPE Kit (Qiagen), according to the manufacturer's protocol. cDNA was synthesized from 500 ng of total RNA using Invitrogen SuperScript IV First-Strand Synthesis System (Thermo-Fisher Scientific) according to the manufacturer's instructions. qPCR was performed with Applied Biosystems SYBR green PCR Master Mix (Thermo-Fisher Scientific). In brief, a 10 µl mixture was used containing 5 µl SYBR Green PCR master mix, 0.5 µl forward and reverse primer, 2.5 µl sterile water, and 2 µl of complementary DNA template. The real-time PCR was performed using Applied Biosystems QuantStudio 3 Real-Time PCR System (Thermo-Fisher Scientific) according to the manufacturer's instructions. Gene-specific primers (**Supplementary Table S2**) for detection and quantification of murine genes investigated in this study were purchased from Integrated DNA Technologies. All data were normalized to 18S rRNA and quantitative measurements were obtained using the $\Delta\Delta$Ct method.

## Statistical analysis

For multiple-group comparisons, data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. A two-tailed Student's *t*-test was used for two-group comparisons. *P* values <0.05 were considered to be statistically significant and values <0.01 were considered highly significant. All statistical analyses were performed using RStudio (version 1.1.463) with the stats and stats4 packages (versions 3.5.1).

# Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s

# Ethics statement

The animal study was reviewed and approved by IACUC from the Lankenau Institute for Medical Research.

# Author contributions

EZ, TK and EHK designed, performed experiments, analyzed, interpreted, and graphed data, and prepared the manuscript. GH provided expert advice and experimental design, and together with PBM interpreted the data, and critically reviewed the manuscript. PBM and JC provided drug development and experimental data, and constructs; YZ and KB carried out all Swiss Webster studies, AA carried out all Micro-CT scanning and QPCR analysis; SB provided animal care and tissue preparation; and KB carried out histology and immunohistochemistry studies. DS provided clinical dental expertise and manuscript preparation. All authors contributed to the article and approved the submitted version.

# Acknowledgments

This work was supported by the grant DE021104 from NIDCR (NIH) and the grant PR180789: W81XWH-19-1-0467 from DOD. EZ and SB were supported by the Stanley and Fiona Druckenmiller Family Foundation.

# Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

# Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of

their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fdmed.2022.992722/full#supplementary-material.

## References


1. Brockes JP, Kumar A. Appendage regeneration in adult vertebrates and implications for regenerative medicine. *Science*. (2005) 310:1919–23. doi: 10.1126/science.1115200

2. Stocum DL, Cameron JA. Looking proximally and distally: 100 years of limb regeneration and beyond. *Dev Dyn*. (2011) 240:943–68. doi: 10.1002/dvdy.22553

3. Sousounis K, Qi F, Yadav MC, Millán JL, Toyama F, Chiba C, et al. A robust transcriptional program in newts undergoing multiple events of lens regeneration throughout their lifespan. *eLife*. (2015) 2:4. doi: 10.7554/eLife.09594

4. D'Aquino R, De Rosa A, Lanza V, Tirino V, Laino L, Graziano A, et al. Human mandible bone defect repair by the grafting of dental pulp stem/progenitor cells and collagen sponge biocomplexes. *Eur Cell Mater*. (2009) 18:75–83. doi: 10.22203/eCM.v018a07

5. La Noce M, Paino F, Spina A, Naddeo P, Montella R, Desiderio V, et al. Dental pulp stem cells: state of the art and suggestions for a true translation of research into therapy. *J Dent*. (2014) 42:761–8. doi: 10.1016/j.jdent.2014.02.018

6. Shi X, Mao J, Liu Y. Pulp stem cells derived from human permanent and deciduous teeth: biological characteristics and therapeutic applications. *Stem Cells*. (2020) 9:445–64. doi: 10.1002/sctm.19-0398

7. Weissman IL. Stem cell: units of development, units of regeneration, and units of evolution. *Cell*. (2000) 100:157–68. doi: 10.1016/S0092-8674(00)81692-X

8. Blanpain C, Fuchs E. Plasticity of epithelial stem cells in tissue regeneration. *Science*. (2014) 344:1242281. doi: 10.1126/science.1242281

9. Mao AS, Mooney DJ. Regenerative medicine: current therapies and future directions. *PNAS*. (2015) 112:14452–9. doi: 10.1073/pnas.1508520112

10. Dahiya P, Kamal R. Hyaluronic acid: a boon in periodontal therapy. *N Am J Med Sci*. (2013) 5:309–15. doi: 10.4103/1947-2714.112473

11. Casale M, Moffa A, Vella P, Sabatino L, Capuano F, Salvinelli B, et al. Hyaluronic acid: perspectives in dentistry. A systematic review. *Int J Immunopath Pharmacol*. (2016) 29:572–82. doi: 10.1177/0394632016652906

12. Derwich M, Mitus-Kenig M, Pawlowska E. Mechanisms of action and efficacy of hylauronic acid, corticosteroids, and platelet-rich plasma in the treatment of TMJ osteoarthritis – A systemic review. *Int J Mol Sci*. (2021) 22:7405. doi: 10.3390/ijms22147405

13. Clark LD, Clark RK, Heber-Katz E. A new murine model for mammalian wound repair and regeneration. *Clin Immunol Immunopathol*. (1998) 88:35–4. doi: 10.1006/clin.1998.4519

14. McBrearty BA, Desquenne-Clark L, Zhang X-M, Blankenhorn EP, Heber-Katz E. Genetic analysis of a mammalian wound healing trait. *Proc Natl Acad Sci USA*. (1998) 95:11792–7. doi: 10.1073/pnas.95.20.11792

15. Edwards RG. From embryonic stem cells to blastema and MRL mice. *Reprod BioMed Online*. (2008) 16:425–61. doi: 10.1016/S1472-6483(10)60605-0

16. Heber-Katz E. Oxygen, metabolism, and regeneration – lessons from mice. *Trends Mol Med*. (2017) 23:1024–36. doi: 10.1016/j.molmed.2017.08.008

17. Heydemann A. The super super-healing MRL mouse strain. *Front Biol*. (2012) 7:522–53. doi: 10.1007/s11515-012-1192-4

18. Heber-Katz E, Messersmith P. Drug delivery and epimorphic salamander-type regeneration. *Adv Drug Deliv Rev*. (2018) 129:254–61. doi: 10.1016/j.addr.2018.02.006

19. Naviaux RK, Le TP, Bedelbaeva K, Leferovich J, Gourevitch D, Sachadyn P, et al. Retained features of embryonic metabolism in the adult MRL mouse. *Mol Genet Metab*. (2009) 96:133–44. doi: 10.1016/j.ymgme.2008.11.164

20. Heber-Katz E, Naviaux R. The MRL mouse: A model of regeneration and cancer. In: NA Berger, editors. *Murine models, energy balance, and cancer*. Springer (2015). p. 47–64.

21. Wang GL, Semenza GL. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc Natl Acad Sci USA*. (1993) 90:4304–8. doi: 10.1073/pnas.90.9.4304

22. Nagao A, Kobayashi M, Koyasu S, Chow CCT, Harada H. HIF-1-Dependent reprogramming of glucose metabolic pathway of cancer cells and its therapeutic significance. *Int J Mol Sci*. (2019) 20:238. doi: 10.3390/ijms20020238

23. Zhang Y, Bedelbaeva K, Strehin I, Gourevitch D, Messersmith PB, Heber-Katz E. Drug-induced regeneration in adult mice. *Sci Transl Med*. (2015) 7:290. doi: 10.1126/scitranslmed.3010228

24. Yoshida GJ. Metabolic reprogramming: the emerging concept and associated therapeutic strategies. *J Exp Clin Cancer Res*. (2015) 34:111. doi: 10.1186/s13046-015-0221-y

25. Jacquet P, Stephanou A. Metabolic reprogramming, questioning, and implications for cancer. *Biology (Basel)*. (2021) 10:129. doi: 10.3390/biology10020129

26. Kim J. Regulation of immune cell functions by metabolic reprogramming. *J Immunol Res*. (2018) 2018:8605471:1–12. doi: 10.1155/2018/8605471

27. Murphy M, O'Neill LAJ. How should we talk about metabolism? *Nat Immunol*. (2020) 21:713–5. doi: 10.1038/s41590-020-0691-8

28. Franklin TJ, Morris WP, Edwards PN, Large MS, Stephenson R. Inhibition of prolyl 4-hydroxylase in vitro and in vivo by members of a novel series of phenanthrolinones. *Biochem J*. (2001) 353(Pt 2):333–8. doi: 10.1042/bj3530333

29. Cheng J, Amin D, Latona J, Heber-Katz E, Messersmith PB. Supramolecular polymer hydrogels for drug-induced tissue regeneration. *ACS Nano*. (2019) 13:5493–501. doi: 10.1021/acsnano.9b00281

30. Latona J, Shah A, Cheng J, Messersmith P, Heber-Katz E. Enhanced liver regeneration after partial hepatectomy in mice treated with a prolyl hydroxylase inhibitor. *Am J Transplant*. (2017) 17(Suppl 3).

31. Abe T, Hajishengallis G. Optimization of the ligature-induced periodontitis model in mice. *J Immunol Methods*. (2013) 394:49–54. doi: 10.1016/j.jim.2013.05.002

32. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol*. (2015) 15:30–44. doi: 10.1038/nri3785

33. Graves DT, Fine D, Teng YT, Van Dyke TE, Hajishengallis G. The use of rodent models to investigate host-bacteria interactions related to periodontal diseases. *J Clin Periodontol*. (2008) 35:89. doi: 10.1111/j.1600-051X.2007.01172.x

34. Pryce BA, Brent AE, Murchison ND, Tabin CJ, Schweitzer R. Generation of transgenic tendon reporters, ScxGFP and ScxAP, using regulatory elements of the scleraxis gene. *Dev Dyn*. (2007) 236:1677–82. doi: 10.1002/dvdy.21179

35. Inoue M, Ebisawa K, Itaya T, Sugito T, Yamawaki-Ogata A, Sumita Y, et al. Effect of GDF-5 and BMP-2 on the expression of tendon/ligamentogenesis-related markers in human PDL-derived cells. *Oral Dis*. (2012) 18:206–12. doi: 10.1111/j.1601-0825.2011.01871.x

36. Rogulic H, Mathews BG, Yang W, Cvija H, Mina M, Kalajzic I. In vivo identification of periodontal progenitor cells. *J Dent Res*. (2013) 92:709–15. doi: 10.1177/0022034513493434

37. Takimoto A, Kawatsu M, Yoshimoto Y, Kawamoto T, Seiryu M, Takano-Yamamoto T. Scleraxis and osterix antagonistically regulate tensile force-responsive remodeling of the periodontal ligament and alveolar bone. *Development*. (2015) 142:787–96. doi: 10.1242/dev.116228

38. Sato O, Maeda T, Kobayashi S, Iwanaga T, Fujita T, Takahashi Y. Innervation of periodontal ligament and dental pulp in the rat incisor: an immuno-histochemical investigation of neurofilament protein and glia-specific S-100 protein. *Cell Tissue Res*. (1987) 251:13–21. doi: 10.1007/BF00215442

39. Tatullo M, Marrelli M, Kevin M, Shakesheff KM, White LJ. Dental pulp stem cells: function, isolation and applications in regenerative medicine. *J Tissue Eng Regen Med*. (2015) 9:1205–627. doi: 10.1002/term.1899

40. Wilson KR, Kang I-H, Baliga U, Xiong Y, Chatterjee S, Moore E, et al. Hematopoietic stem cells as a novel source of dental tissue cells. *Sci Rep*. (2018) 8:8026. doi: 10.1038/s41598-018-26258-y

41. Leonardi R, Lanteri E, Stivala F, Travali S. Immunolocalization of CD44 adhesion molecules in human periradicular lesions. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. (2000) 89:480–5. doi: 10.1016/S1079-2104(00)70129-6

42. Leonardi R, Loreto C, Caltabiano R, Caltabiano C. Immunolocalization of CD44s in human teeth. *Acta Histochem*. (2006) 108:425–9. doi: 10.1016/j.acthis.2006.06.006

43. Merzel J, Salmon CR. Growth and the modeling/remodeling of the alveolar bone of the rat incisor. *Anat Rec*. (2008) 291:827–34. doi: 10.1002/ar.20703

44. Dontas IA, Tsolakis AI, Khaldi L, Patra E, Lyritis GP. Malocclusion in aging wistar rats. *JAALAS*. (2010) 49:22–6.

45. Eke PI, Dye BA, Wei L, Thornton-Evans GO, Genco RJ. Prevalence of periodontitis in adults in the United States: 2009 and 2010. *J Dent Res*. (2012) 91:914–23. doi: 10.1177/0022034512457373

46. Chapple IL. Time to take periodontitis seriously. *Br Med J*. (2014) 348:g2645. doi: 10.1136/bmj.g2645

47. Beikler T, Flemmig TF. Oral biofilm-associated diseases: trends and implications for quality of life, systemic health and expenditures. *Periodontol 2000*. (2011) 55:87–103. doi: 10.1111/j.1600-0757.2010.00360.x

48. Casanova L, Hughes F, Preshaw P. Diabetes and periodontal disease. *BDJ Team*. (2015) 1:15007. doi: 10.1038/bdjteam.2015.7

49. Mealey BL. Periodontal disease and diabetes: a two-way street. *JADA*. (2006) 147:526–31. doi: 10.14219/jada.archive.2006.0404

50. Foster BL, Somerman MJ. Regenerating the periodontium: is there a magic formula? *Orthod Craniofac Res*. (2005) 8:285–91. doi: 10.1111/j.1601-6343.2005.00351.x

51. Murakami S. Periodontal tissue regeneration by signaling molecule(s): what role does basic fibroblast growth factor (FGF-2) have in periodontal therapy? *Periodontol 2000*. (2011) 56:188–208. doi: 10.1111/j.1600-0757.2010.00365.x

52. Tan M, Gu Q, He H, Parmarthy D, Semenza GL, Sun Y. SAG/ROC2/RBX2 is a HIF-1 target gene that promotes HIF-1α ubiquination and degradation. *Oncogene*. (2008) 27:1404–11. doi: 10.1038/sj.onc.1210780

53. Cheverud JM, Lawson HA, Bouckaert K, Kossenkov A, Showe L, Cort L, et al. Fine-mapping quantitative trait loci affecting murine external ear tissue regeneration in the LG/J by SM/J advanced intercross line. *Heredity*. (2014) 112:508–18. doi: 10.1038/hdy.2013.133

54. Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, et al. The homeoprotein nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*. (2003) 113:631–42. doi: 10.1016/S0092-8674(03)00393-3

55. Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet*. (2000) 24:372–6. doi: 10.1038/74199

56. Dunwoodie SL. The role of hypoxia in development of the mammalian embryo. *Dev Cell*. (2009) 17:755–73. doi: 10.1016/j.devcel.2009.11.008

57. Mohyeldin A, Garzon-Muvdi T, Quinones-Hinojosa A. Oxygen in stem cell biology: a critical component of the stem cell niche. *Cell Stem Cell*. (2010) 7:150–61. doi: 10.1016/j.stem.2010.07.007

58. Mathieu J, Zhang Z, Zhou W, Wang AJ, Heddleston JM, Pinna CM, et al. HIF Induces human embryonic stem cell markers in cancer cells. *Cancer Res*. (2011) 71:4640–52. doi: 10.1158/0008-5472.CAN-10-3320

59. Ehnes DD, Hussein AM, Ware CB, Mathieu J, Ruohola-Baker H. Combinatorial metabolism drives the naïve to primed pluripotent chromatin landscape. *Exp Cell Res*. (2020) 389(2):111913. doi: 10.1016/j.yexcr.2020.111913

60. Shyh-Chang N, Daley GQ, Cantley LC. Stem cell metabolism in tissue development and aging. *Development*. (2013) 140:2535–47. doi: 10.1242/dev.091777

61. Axelson H, Fredlund E, Ovenberger M, Landberg G, Pahlman S. Hypoxia-induced dedifferentiation of tumor cells – a mechanism behind heterogeneity and aggressiveness of solid tumors. *Semin Cell Dev Biol*. (2005) 16:554–63. doi: 10.1016/j.semcdb.2005.03.007

62. Call MK, Grogg MW, Tsonis PA. An eye on regeneration. *Anat Rec B New Anat*. (2005) 287:42–8. doi: 10.1002/ar.b.20084

63. Nagai K, Ideguchi H, Kajikawa T, Xiaofei Li X, Chavakis T, Cheng J, et al. An injectable hydrogel-formulated inhibitor of prolyl-4-hydroxylase promotes T regulatory cell recruitment and enhances alveolar bone regeneration during resolution of experimental periodontitis. *FASEB J*. (2020):1–15 34:13726–40. doi: 10.1096/fj.202001248R

64. Kemoun P, Laurencin-Dalicieux S, Rue J, Vaysse F, Romeas A, Arzate H, et al. Localization of STRO-1, BMP-2/-3/-7, BMP receptors and phosphorylated smad-1 during the formation of mouse periodontium. *Tissue Cell*. (2007) 39:257–66. doi: 10.1016/j.tice.2007.06.001

65. Chen SC, Marino V, Gronthos S, Bartold PM. Location of putative stem cells in human periodontal ligament. *J Periodontal Res*. (2006) 41:547–53. doi: 10.1111/j.1600-0765.2006.00904.x

66. Xu J, Wang W, Kapila Y, Lotz J, Kapila S. Multiple differentiation capacity of STRO-1+/CD146+ PDL mesenchymal progenitor cells. *Stem Cells Dev*. (2009) 18:487–96. doi: 10.1089/scd.2008.0113

67. San Miguel SM, Fatahi MR, Li H, Igwe JC, Aguila HL, Kalajzic I. Defining a visual marker of osteoprogenitor cells within the periodontium. *J Periodontal Res*. (2010) 45:60–70. doi: 10.1111/j.1600-0765.2009.01201.x

68. Mrozik K, Gronthos S, Shi S, Bartold PM. A method to isolate, purify, and characterize human periodontal ligament stem cells. *Methods Mol Biol*. (2010) 666:269–84. doi: 10.1007/978-1-60761-820-1_17

69. McCulloch CA. Progenitor cell populations in the periodontal ligament of mice. *Anat Rec*. (1985) 211:258–62. doi: 10.1002/ar.1092110305

70. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet*. (2004) 364:149–55. doi: 10.1016/S0140-6736(04)16627-0

71. Shi S, Bartold PM, Miura M, Seo BM, Robey PG, Gronthos S. The efficacy of mesenchymal stem cells to regenerate and repair dental structures. *Orthod Craniofac Res*. (2005) 8:191–9. doi: 10.1111/j.1601-6343.2005.00331.x

72. Kaku M, Komatsu Y, Mochida Y, Yamauchi M, Mishina Y, Ko CC. Identification and characterization of neural crest-derived cells in adult periodontal ligament of mice. *Arch Oral Biol*. (2012) 57:1668–75. doi: 10.1016/j.archoralbio.2012.04.022

73. Volponi AA, Pang Y, Sharpe PT. Stem cell-based biological tooth repair and regeneration. *Trends Cell Biol*. (2010) 20:715–22. doi: 10.1016/j.tcb.2010.09.012

74. Cohen F-M, Sun H-H, Lu H, Yu Q. Stem cell-delivery therapeutics for periodontal tissue regeneration. *Biomaterials*. (2012) 33:6320–44. doi: 10.1016/j.biomaterials.2012.05.048

75. Hosoya A, Nakamura H, Ninomiya T, Yoshiba K, Yoshiba N, Nakaya H, et al. Immunohistochemical localization of a-smooth muscle actin during rat molar tooth development. *J Histochem Cytochem*. (2006) 54:1371–8. doi: 10.1369/jhc.6A6980.2006

76. Shetty H, Kakade A, Shetty S, Neelakantan NS, Desai R, Beri K. Immuno histochemical characterization of stem cell and differentiation markers of the dental pulp of human natal teeth. *Future Sci OA*. (2018) 4:FS0342. doi: 10.4155/fsoa-2018-0062

77. Einhorn TA, Gerstenfeld L. Fracture healing: mechanisms and interventions. *Nat Rev Rheumatol*. (2015) 11:45–54. doi: 10.1038/nrrheum.2014.164

78. Galatz LM, Gerstenfeld LC, Heber-Katz E, Sa R. Tendon regeneration and scar formation: the concept of scarless healing. *J Orthopedic Surg*. (2015) 33:823–31. doi: 10.1002/jor.22853

79. Elmer Perkin. (2013). Quantum FX: Low Dose Computed Tomography [Product Note]. Available at: https://www.perkinelmer.com/labsolutions/resources/docs/BRO_010486_01_PRD_QuantumFX.pdf

80. Li CH, Amar S. Morphometric, histomorphometric, and micro-computed tomographic analysis of periodontal inflammatory lesions in a murine model. *J Periodontol*. (2007) 78:1120. doi: 10.1902/jop.2007.060320

frontiers | Frontiers in Dental Medicine

TYPE Perspective
PUBLISHED 10 January 2023
DOI 10.3389/fdmed.2022.1074339



OPEN ACCESS

EDITED BY
Francisco Nociti,
Universidade Estadual de Campinas, Brazil

REVIEWED BY
Marcelo Henrique Napimoga,
São Leopoldo Mandic School, Brazil
Renato Correa Viana Casarin,
Universidade Estadual de Campinas, Brazil

*CORRESPONDENCE
Sukirth M. Ganesan
✉ sukirth-ganesan@uiowa.edu

SPECIALTY SECTION
This article was submitted to Systems Integration, a section of the journal Frontiers in Dental Medicine

RECEIVED 19 October 2022
ACCEPTED 20 December 2022
PUBLISHED 10 January 2023

CITATION
Fraser D and Ganesan SM (2023) Microbiome, alveolar bone, and metabolites: Connecting the dots.
Front. Dent. Med 3:1074339.
doi: 10.3389/fdmed.2022.1074339



# Microbiome, alveolar bone, and metabolites: Connecting the dots

David Fraser[1] and Sukirth M. Ganesan[2]*

[1]National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, United States, [2]Department of Periodontics, College of Dentistry, University of Iowa, Iowa City, IA, United States

The oral microbiome (OM) is a diverse and dynamic collection of species, separated from alveolar bone by the oral mucosa. Pathogenic shifts in the OM (dysbiosis) during periodontitis are associated with an inflammatory response in the oral mucosa that drives alveolar bone resorption. Alveolar bone is also affected by metabolic disorders such as osteoporosis. Accumulating evidence has linked another microbial community, the gut microbiome (GM), to systemic bone metabolism and osteoporosis. Underlying this connection is the biologic activity of metabolites, byproducts of host and bacterial activity. Limited evidence also suggests that metabolites in the oral cavity signal between the OM and immune system, influencing both alveolar bone homeostasis and pathologic bone destruction in periodontitis. While the oral cavity and gut are connected through the gastrointestinal tract, dissimilar roles for known metabolites between these two niches exemplify the difficulty in translating knowledge on gut-derived metabolites and bone metabolism to alveolar bone. Integrated metabolomic, transcriptomic, and metagenomic approaches hold promise for resolving these challenges and identifying novel metabolites which impact alveolar bone health. Further interrogation through mechanistic testing in pre-clinical models and carefully controlled clinical studies have potential to lead toward translation of these discoveries into meaningful therapies.

KEYWORDS
alveolar bone, oral microbiome, gut microbiome, metabolites, periodontitis, osteoporosis

## Introduction

The human body is colonized by trillions of microbes (1). Recent advances, including the Human Microbiome Project and the development of next generation sequencing technologies, have convincingly demonstrated that distinct microbial communities colonize different body sites and interact with host cells to modulate health and disease (2, 3). It is further established that maintenance of health requires a state of homeostasis between the microbiome and immune system across different body sites, also known as niches (4, 5). Two distinct niches, the gut and oral cavity, are characterized by a complex relationship between the host and gut microbiome (GM) and oral microbiome (OM), respectively (6, 7). Disturbances in these homeostatic interactions drive dysbiosis and inflammation and are associated with

several chronic diseases, including inflammatory bowel disease (IBD), type 2 diabetes (T2D), obesity, metabolic syndrome, osteoporosis, rheumatoid arthritis, Alzheimer's disease, periodontal disease, dental caries, and various cancers (8, 9). The role of the OM in driving alveolar bone destruction is well established (10), and a role for the GM in regulating systemic bone health has become increasingly appreciated (11, 12). Accordingly, the nature of the microbial-host interrelationships that regulate bone metabolism in health and disease are active areas of investigation.

Alveolar bone is the specialized portion of the mandible and maxilla which houses, supports, and protects the root structures of teeth (13). Formation and remodeling of alveolar bone is shaped by local factors, such as the eruption of teeth into the oral cavity and ongoing masticatory forces, and systemic regulation through hormonal and metabolic signaling (14, 15). Distinct from other skeletal structures, alveolar bone lies in close proximity to OM biofilms and undergoes resorption during the course of periodontitis, a chronic and widespread disease (16). The periodontitis-associated OM is characterized by dysbiotic biofilms on tooth and root surfaces containing several pathogenic species such as *P. gingivalis, Treponema denticola, Tannerella forsythia*, and *A. actinomycetemcomitans* (17). Concurrently, a heavy immune cell infiltration is present in the gingiva, the oral mucosal tissue surrounding the teeth, which drives osteoclast activity in the underlying alveolar bone (18, 19). Diseases that affect bone metabolism, such as osteoporosis, also affect alveolar bone health (20). Thus, an interplay between OM, the associated immune response, and local and systemic factors affecting bone shape the pathogenesis of alveolar bone loss.

## Metabolites, the currency of bacterial-host crosstalk

Metabolites are the byproducts of microbial or host metabolism specific to the environment, modulating health by signaling to host cells and influencing bacterial community interactions (21). Host amino acids (22) and byproducts from glucose-related pathways (e.g., glycolysis and gluconeogenesis) (23) and mitochondrial metabolism (e.g., tricarboxylic acid cycle metabolites succinate, fumarate, and aconitate) (24) have well known roles in signaling within and between immune and bone cell populations. Microbial metabolite production, best characterized in the gut environment, is heavily driven by dietary intake, with fermentation of complex carbohydrates and proteins leading to production of short- and branched-chain fatty acids, and metabolism of proteins and peptides producing amines, phenols, and indoles from amino acids (25, 26).

Interactions between microbially-derived metabolites and host cells are increasingly recognized as drivers of human health and disease (21, 27). Extensive research in the gut has identified roles for microbially derived metabolites, including secondary bile acids, short-chain fatty acids (SCFAs), trimethylamine-N-oxide (TMAO), polysaccharide A, 4-ethyl phenyl sulfate, and catecholamines, in systemic diseases affecting bone (28–31). In contrast, there is currently a narrower understanding of the scope and nature of OM-derived metabolites and their role in alveolar bone health (**Figure 1**).

The emerging field of metabolomics has enabled cataloging of both well-known and novel metabolites using an array of platforms and techniques (32). The number of metabolite entries in the Human Metabolome Database, the most comprehensive collection of human metabolites, has burgeoned from 2,180 entries in 2007 to 217,920 annotated metabolite entries and 1,581,537 unannotated entries (33). These technologic advances in unbiased metabolomics have significant potential to (1) uncover the net biological activity in the oral cavity, (2) expand our knowledge of the pathogenesis of alveolar bone destruction beyond identifying specific bacterial species, and (3) identify novel targets for disease diagnosis, prognosis, and treatment.

## Lessons learned from the gut microbiome

The microbiome colonizing the human intestine, known as the gut microbiome (GM), is the largest microbial niche in the human body and comprises a complex ecosystem with established roles in human health and disease (34). Initially formed *in utero* or at birth, the GM rapidly develops between ages 1–4 and continues to evolve in response to intrinsic and environmental factors such as geographic location, gender, diet, and antibiotic use (35). The intestinal mucosal epithelium serves as the interface between host and the microbiome, controlling interactions through the coordinated activities of mucus, epithelial cell junctions, immunoglobulin A, antimicrobial peptides, and immune cells (36, 37). Nutrients and metabolites also pass through this barrier to interact with local cells or enter the circulation (38).

Bone remodeling and homeostasis are regulated by a network of systemic hormones, including parathyroid hormone (PTH), calcitonin, FGF23, 1,25-dihydroxyvitamin $D_3$ (Vitamin D), and estrogen. The GM is considered an endocrine organ (39) and animal models show that altering or preventing GM development influences skeletal bone mass and osteoclast activity (40–42). Gut microbes synthesize vitamin $K_2$ which stimulates osteoblast activity and is a cofactor for post-translational modification of osteocalcin (12). Disruption of the ecosystem with antibiotics inhibits Vitamin $K_2$ synthesis and reduces bone quality (43). Enzymes secreted by gut microbiota can metabolize or re-activate estrogen, altering circulating or excreted levels (44). GM dysbiosis can also mediate estrogen deficiency-related bone loss through

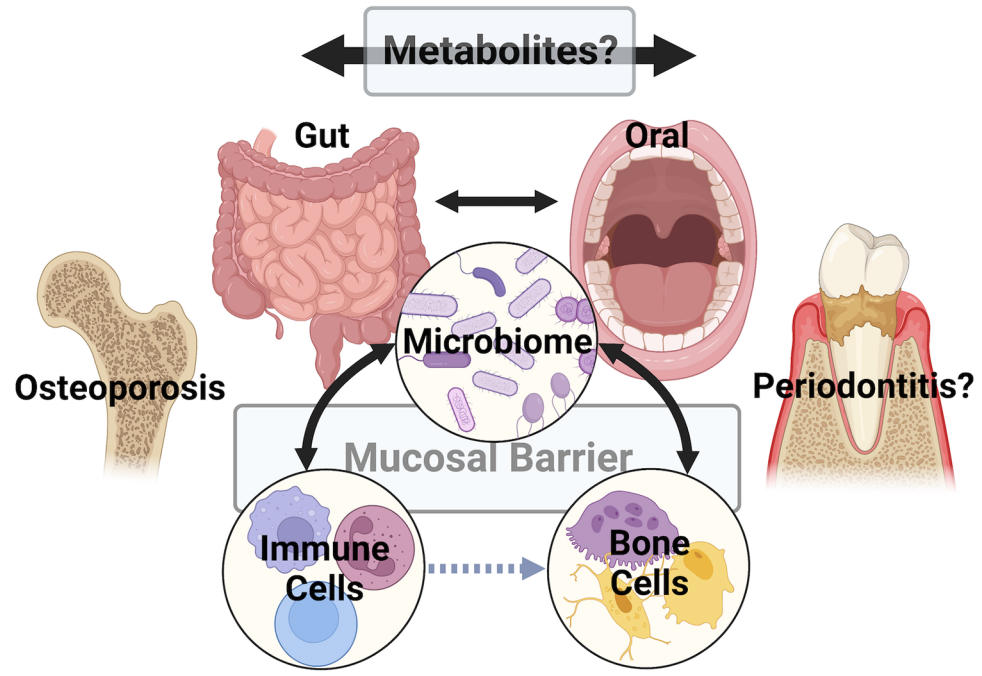



FIGURE 1
A multitude of studies have explored the connections between the gut and/or oral microbiomes, the host immune system, and bone cells (i.e., osteoblasts, osteocytes, and osteoclasts). Recent work suggests that metabolites are key signaling factors in these pathways (represented by black bidirectional arrows), acting directly or indirectly (i.e. *via* the immune system – gray dashed arrow) to influence pathologic bone disorders like osteoporosis. A significant challenge is translating knowledge gained from studies of the gut and osteoporosis to the oral cavity to understand if metabolites play similar or distinct roles in the metabolism of alveolar bone. Underlying this challenge are differences in mucosal barrier structures, microbiome populations, and immune cells between the gut and oral mucosa. Figure created with BioRender.com.

increases in mucosal permeability, immune cell numbers, and inflammatory cytokines (45, 46).

The GM can enable serotonin production by enterochromaffin cells (47) and deconjugate bile acid compounds and further metabolize them to secondary bile acids such as lithocholic and deoxycholic acid (48). Gut-derived serotonin may inhibit bone formation (49) and lithocholic acid can bind vitamin D receptor (VDR), leading to inactivation of vitamin D and decreased intestinal calcium absorption (50). Bile acids can also signal enteroendocrine cells to release GLP-1 which promotes bone formation and inhibits bone resorption (51). Hydrogen sulfide ($H_2S$) is produced by gastrointestinal cells and the GM (52). Loss of $H_2S$ results in osteopenia in mice (53) and administration of an $H_2S$ donating compound in ovariectomy-treated mice improves bone formation (54).

Emerging evidence points to gut-derived short-chain fatty acids (SCFAs) as modulators of systemic health and bone maintenance [see reviews (11, 12, 55, 56)]. In brief, SCFAs, including butyrate, propionate, and acetate, are primarily produced by microbial fermentation of non-digestible polysaccharides and are rapidly absorbed through intestinal mucosa, acting as a source of energy for both host and microbiota (56). While SCFAs can directly suppress osteoclast activity and promote osteoblast differentiation (57, 58), signaling between SCFA and endocrine organs or immune cells may underly the connection between GM and bone. Gut microbial colonization or SCFA supplementation is associated with the production of insulin-like growth factor 1 (IFG-1), an important hormone for skeletal growth and bone mass maintenance (59). SCFAs, including butyrate, promote proliferation and differentiation of regulatory T cells (Treg) (60) which may reduce bone absorption by interfering with osteoclast development and activity (61). Butyrate can increase Treg numbers in the intestine and bone marrow which signals to CD8+ T cells to produce WNT10b, a bone anabolic signaling factor (62). Butyrate produced by GM may also regulate PTH-mediated bone formation through signaling in dendritic cells and Tregs (63).

Probiotics have been widely studied as a means to target osteoporosis *via* manipulation of the GM (64). A clinical study showed *Lactobaciillus reuteri* probiotics increased BMD and elevated butyrylcarnitine, which can act as pool and transporter of butyrate (65). Prebiotics are non-digestible oligosaccharides that are selectively fermented in the colon and support growth of specific bacterial species. Positive results for prebiotics in animal models, including increased calcium absorption and improved BMD and bone strength,

have been primarily attributed to fermentation of prebiotics to SCFAs by GM (66). Clinical trials have further indicated that prebiotics can increase intestinal calcium absorption (67).

Altogether, the GM plays a critical role in regulating systemic bone metabolism, in part, through production of metabolites. GM-derived metabolites act both locally and systemically on host cells to drive immune responses that shape bone metabolism. Improved understanding of GM metabolites and their role in shaping bone health have led to development of therapeutic interventions, including probiotics and prebiotics, suggesting that probing the connection between the oral cavity and gut and identifying similar pathways in the oral cavity has promise for improving alveolar bone health.

## The oral-gut-bone connection

Ingested saliva, food, and drink directly connect the OM and GM (68, 69). Patients with conditions characterized by GM inflammation and dysbiosis, such as inflammatory bowel disease, have an altered OM, increased numbers of OM-derived species in the gut, and higher rates of periodontitis (70, 71). Studies in mice suggest that ingested OM bacteria can reach the gut and induce an inflammatory immune response (72), and immune cells exposed to OM can reach the gut to interact with OM-derived gut microbes (73). Gut colonization with specific bacterial species can also influence T cell development in alveolar bone marrow and increase alveolar bone osteoclast activity, further illustrating the potential bidirectional mechanisms whereby microbial populations in both gut and oral cavity can help disrupt or maintain bone homeostasis (74).

Studies probing the oral-gut connection have shown that adminstering oral *P. gingivalis* modifies the GM and alters serum and gut metabolite profiles (75, 76), including increasing gut lactic acid and reducing succinic acid and butyrate levels (77). Additional evidence connecting GM, metabolites, and alveolar bone has been provided by animal studies of probiotic administration or diet alterations. In ovariectomized rats, probiotics increased levels of butyrate-producing GM and reduced osteoclast and Th17 cell numbers while increasing Treg cells and minimizing maxillary bone loss during ligature-induced periodontitis (78). Transplantation of fecal contents from high fat diet (HFD) obese mice altered host GM and gut and serum metabolite compositions with little change in the OM while increasing Th17 cells in submandibular and mesenteric lymph nodes and aggravating alveolar bone loss in experimental periodontitis (79). One metabolite of purine degradation, uric acid, was increased in serum with HFD fecal transplant and induction of periodontitis, and administration of allopurinol suppressed alveolar bone destruction in uremic mice (79).

Overall, these findings lend support to the concept that the oral health is connected to systemic health and highlight distinct molecular pathways connecting the gut and oral microbiomes and the immune system through metabolites. Whether such mechanisms identified in mouse models can be translated to meaningful interventions in humans is still unknown. Nevertheless, such studies provide further motivation for studying the role of metabolites in bone health and, in particular, within the oral niche.

## Oral metabolites and alveolar bone

The oral cavity is rich in byproducts of host and OM metabolism (80). Saliva and gingival crevicular fluid (GCF) show distinct profiles of metabolite compositions between health and periodontitis (81–83) with clinical studies showing specific associations between periodontitis and increased levels of arachidonic acid, purine, pyrimidine, glutathione, and amino acid metabolites (84–87). Accordingly, various metabolites have been explored as predictors of gingival inflammation or periodontitis (88) or as factors that regulate the disruption or maintenance of the gingival epithelial barrier (junctional epithelium) (89). However, clear evidence is lacking for how specific metabolites or metabolic pathways act to help maintain alveolar bone in oral health or aggravate bone destruction during periodontitis.

Existing studies on oral metabolites and alveolar bone have focused on butyrate, and contrary to the gut, have ascribed it a pathogenic role in periodontitis (**Figure 2**). This distinction may be due to several factors, including differences in butyrate concentrations, mucosal tissue structure, and microbial populations between GM and OM environments (90, 91). Periodontitis-associated oral bacteria, *P. gingivalis* and *F. nucleatum*, produce butyrate (92). Further, butyrate can stimulate heme production which supports growth of periodontal pathogens like *P. gingivalis* (90). Butyrate concentrations in periodontal pockets can reach up to 14 mM (93) with levels correlating to periodontal disease severity (94) and decreasing in GCF after periodontal treatment (95). While butyrate levels may be similar or higher in the colon compared to the oral cavity, a much lower concentration may actually reach colonic epithelial cells after penetrating through the thick colon mucous layer (96). A recent animal study found that butyrate could disrupt the periodontal junctional epithelial barrier (97). This finding, coupled with *in vitro* studies showing a negative effect of butyrate on different oral cell types (90, 91), and in particular, epithelial cells (98), suggests that differences in the mucosal barrier anatomy between gut and periodontal tissues could account for some of the opposing effects of butyrate on alveolar vs. other bone sites.

Conceivably, OM-derived butyrate and other SCFAs signal to immune, epithelial, and stromal cells in periodontal tissues

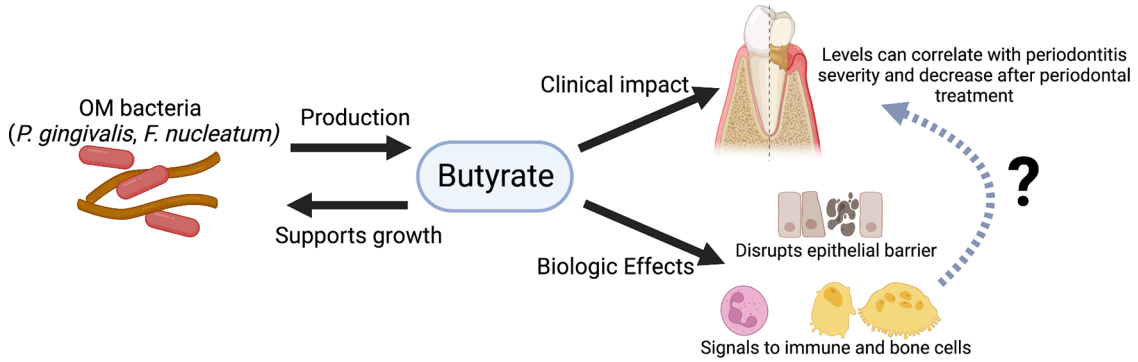



FIGURE 2
Graphical summary of evidence for butyrate's role in periodontitis. Periodontitis-associated bacteria found within the OM (e.g., *P. gingivalis* and *F. nucleatum*) produce butyrate, which in turn can support their growth. Clinical studies have shown an association between butyrate levels and periodontitis and found that butyrate levels decrease after periodontal treatment. Experimental studies indicate butyrate may disrupt the junctional epithelial barrier and can signal to immune and bone cells. However, the exact mechanisms connecting butyrate and its possible biologic effects to periodontitis and alveolar bone are still unknown. Figure created with BioRender.com.

which could then interact with osteoblasts and osteoclasts. SCFAs appear to affect the ability of neutrophils to respond to the periodontal pathogen *A. actinomycetemcomitans* (99). Mice deficient in the SCFA receptor FFAR2 showed increased alveolar bone loss and decreased maxillary bone density, with the latter partially rescued by a high fiber diet (100). While osteoclasts derived from FFAR2-deficient mice showed increased *in vitro* differentiation, the only SCFA which could inhibit this activity was butyrate, indicating that butyrate acted independently of the FFAR2 receptor.

Clearly, further work is needed to identify how metabolites beyond SCFAs affect alveolar bone and to better understand how butyrate and other metabolites modulate alveolar bone metabolism through the oral mucosal immune response to OM biofilms. Additional questions inspired by the role of GM metabolites in bone health may provide insight. Do metabolites produced in the oral cavity act on the oral mucosal immune system similar to how the GM indirectly influences bone health? Do differences or similarities between the oral and gut niches underly the impact of oral metabolites on alveolar bone? Answers to these and other questions, aided by advances in scientific techniques, may provide new options for diagnosing, treating, or preventing periodontitis and the associated loss of alveolar bone.

## The path forward

The bulk of studies on periodontitis and alveolar bone thus far continue to focus on OM characterization through either 16S or whole genome shotgun sequencing approaches and interrogating the host immune response. Work investigating the biologically active small compounds that determine the net functional activity in the oral environment remains scarce. However, such investigations are beginning to emerge, enabled by technological advances in metabolomics. Indeed, recent studies have demonstrated that combining metabolomics with transcriptomics, 16S DNA genomics, and other unbiased techniques has potential for identifying new molecular pathways and therapeutic targets for periodontitis and alveolar bone loss (101–103).

In parallel, rigorous studies are required for determining the mechanisms behind oral metabolites and alveolar bone. The majority of existing studies on oral metabolites utilize *in vitro* models of homogeneous cells and/or bacterial populations. Such approaches have significant limitations in their ability to recapitulate the complex environment of subgingival biofilms, oral mucosal tissues, and underlying alveolar bone. Thus, carefully controlled animal studies should be designed to investigate the mechanisms behind host and bacterial metabolites and alveolar bone health.

The translation of findings on known or novel oral metabolites to effective therapies for maintaining alveolar bone face specific challenges in study design and analysis. Characterization and validation of possible targets for therapy will entail clinical studies with rigorous study design, careful cohort stratification, and inclusion and exclusion criteria to ensure application and reproducibility. Data integration and analysis with multi-omics approaches is challenging due to heterogeneity in the data format from each -omics technologies, discrepancies in annotation, and non-uniform missing data from different data. Additionally, the computational complexity and lack of standardization for analytical and bioinformatic pipelines may hinder reproducibility across studies. Thus, the introduction of standardized protocols for clinical studies and computational approaches, along with techniques to accommodate for data heterogeneity and missing data, are critical for the success of future work. With these tools in hand, an integrative multi-

omics approach combining metabolomics, metagenomics, transcriptomics, and other -omics techniques may be able to resolve the interconnected roles of the OM and immune response in alveolar bone health and disease.

## Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

## Author contributions

DF and SMG: conceptualization, writing – original draft, writing – review and editing. SMG: funding acquisition. All authors contributed to the article and approved the submitted version.

## Funding

DF was supported by intramural programs of the NIH/NIDCR (DIR OCD Clinical Research Fellowship grant no. DE000727). SMG was supported by extramural grant NIH/NIDCR grant no. R03DE030527.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

## References

1. Sender R, Fuchs S, Milo R. Revised estimates for the number of human and Bacteria cells in the body. *PLoS Biol.* (2016) 14(8):e1002533. doi: 10.1371/journal.pbio.1002533

2. Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nat.* (2007) 449(7164):804–10. doi: 10.1038/nature06244

3. Wensel CR, Pluznick JL, Salzberg SL, Sears CL. Next-generation sequencing: insights to advance clinical investigations of the microbiome. *J Clin Invest.* (2022) 132(7):e154944. doi: 10.1172/JCI154944

4. Runge S, Rosshart SP. The mammalian metaorganism: a holistic view on how microbes of all kingdoms and niches shape local and systemic immunity. *Front Immunol.* (2021) 12. doi: 10.3389/fimmu.2021.702378

5. Louca S, Polz MF, Mazel F, Albright MBN, Huber JA, O'Connor MI, et al. Function and functional redundancy in microbial systems. *Nat Ecol Evol.* (2018) 2(6):936–43. doi: 10.1038/s41559-018-0519-1

6. Suárez LJ, Arboleda S, Angelov N, Arce RM. Oral versus gastrointestinal mucosal immune niches in homeostasis and allostasis. *Front Immunol.* (2021) 12. doi: 10.3389/fimmu.2021.705206

7. Radaic A, Kapila YL. The oralome and its dysbiosis: new insights into oral microbiome-host interactions. *Comput Struct Biotechnol J.* (2021) 19:1335–60. doi: 10.1016/j.csbj.2021.02.010

8. Carding S, Verbeke K, Vipond DT, Corfe BM, Owen LJ. Dysbiosis of the gut microbiota in disease. *Microb Ecol Health Dis.* (2015) 26(1):26191. doi: 10.3402/mehd.v26.26191

9. Kumar PS. From focal sepsis to periodontal medicine: a century of exploring the role of the oral microbiome in systemic disease. *J Physiol.* (2017) 595 (2):465–76. doi: 10.1113/JP272427

10. Scannapieco FA, Dongari-Bagtzoglou A. Dysbiosis revisited: understanding the role of the oral microbiome in the pathogenesis of gingivitis and periodontitis: a critical assessment. *J Periodontol.* (2021) 92 (8):1071–8. doi: 10.1002/JPER.21-0120

11. Behera J, Ison J, Tyagi SC, Tyagi N. The role of gut microbiota in bone homeostasis. *Bone.* (2020) 135:115317. doi: 10.1016/j.bone.2020.115317

12. Cooney OD, Nagareddy PR, Murphy AJ, Lee MKS. Healthy gut, healthy bones: targeting the gut microbiome to promote bone health. *Front Endocrinol.* (2021) 11. doi: 10.3389/fendo.2020.620466

13. Nanci A, Bosshardt DD. Structure of periodontal tissues in health and disease. *Periodontol.* (2006) 40:11–28. doi: 10.1111/j.1600-0757.2005.00141.x

14. Jonasson G, Skoglund I, Rythén M. The rise and fall of the alveolar process: dependency of teeth and metabolic aspects. *Arch Oral Biol.* (2018) 96:195–200. doi: 10.1016/j.archoralbio.2018.09.016

15. Sodek J, McKee MD. Molecular and cellular biology of alveolar bone. *Periodontol.* (2000) 24(1):99–126. doi: 10.1034/j.1600-0757.2000.2240106.x

16. Bartold PM, Cantley MD, Haynes DR. Mechanisms and control of pathologic bone loss in periodontitis. *Periodontol.* (2010) 53(1):55–69. doi: 10.1111/j.1600-0757.2010.00347.x

17. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent Jr RL. Microbial complexes in subgingival plaque. *J Clin Periodontol.* (1998) 25(2):134–44. doi: 10.1111/j.1600-051X.1998.tb02419.x

18. Moutsopoulos NM, Konkel JE. Tissue-specific immunity at the oral mucosal barrier. *Trends Immunol.* (2018) 39(4):276–87. doi: 10.1016/j.it.2017.08.005

19. Ikeuchi T, Moutsopoulos NM. Osteoimmunology in periodontitis; a paradigm for Th17/IL-17 inflammatory bone loss. *Bone.* (2022) 167:116500. doi: 10.1016/j.bone.2022.116500

20. Yu B, Wang C-Y. Osteoporosis and periodontal diseases – an update on their association and mechanistic links. *Periodontol.* (2022) 89(1):99–113. doi: 10.1111/prd.12422

21. Blacher E, Levy M, Tatirovsky E, Elinav E. Microbiome-modulated metabolites at the interface of host immunity. *J Immun.* (2017) 198(2):572–80. doi: 10.4049/jimmunol.1601247

22. Kelly B, Pearce EL. Amino assets: how amino acids support immunity. *Cell Metab*. (2020) 32(2):154–75. doi: 10.1016/j.cmet.2020.06.010

23. Karner CM, Long F. Glucose metabolism in bone. *Bone*. (2018) 115:2–7. doi: 10.1016/j.bone.2017.08.008

24. Martínez-Reyes I, Chandel NS. Mitochondrial TCA cycle metabolites control physiology and disease. *Nat Commun*. (2020) 11(1):102. doi: 10.1038/s41467-019-13668-3

25. Oliphant K, Allen-Vercoe E. Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. *Microbiome*. (2019) 7(1):91. doi: 10.1186/s40168-019-0704-8

26. Krautkramer KA, Fan J, Bäckhed F. Gut microbial metabolites as multi-kingdom intermediates. *Nat Rev Microbiol*. (2021) 19(2):77–94. doi: 10.1038/s41579-020-0438-4

27. Kim CH. Immune regulation by microbiome metabolites. *Immunol*. (2018) 154(2):220–9. doi: 10.1111/imm.12930

28. Brown JM, Hazen SL. The gut microbial endocrine organ: bacterially derived signals driving cardiometabolic diseases. *Annu Rev Med*. (2015) 66(1):343–59. doi: 10.1146/annurev-med-060513-093205

29. Dasgupta S, Erturk-Hasdemir D, Ochoa-Reparaz J, Reinecker H-C, Kasper Dennis L. Plasmacytoid dendritic cells mediate anti-inflammatory responses to a gut commensal molecule via both innate and adaptive mechanisms. *Cell Host Microbe*. (2014) 15(4):413–23. doi: 10.1016/j.chom.2014.03.006

30. Asano Y, Hiramoto T, Nishino R, Aiba Y, Kimura T, Yoshihara K, et al. Critical role of gut microbiota in the production of biologically active, free catecholamines in the gut lumen of mice. *Am J Physiol Gastrointest Liver Physiol*. (2012) 303(11):G1288–95. doi: 10.1152/ajpgi.00341.2012

31. Zhou T, Heianza Y, Chen Y, Li X, Sun D, DiDonato JA, et al. Circulating gut microbiota metabolite trimethylamine N-oxide (TMAO) and changes in bone density in response to weight loss diets: the POUNDS lost trial. *Diabetes Care*. (2019) 42(8):1365–71. doi: 10.2337/dc19-0134

32. Bauermeister A, Mannochio-Russo H, Costa-Lotufo LV, Jarmusch AK, Dorrestein PC. Mass spectrometry-based metabolomics in microbiome investigations. *Nat Rev Microbiol*. (2022) 20(3):143–60. doi: 10.1038/s41579-021-00621-9

33. Wishart DS, Guo A, Oler E, Wang F, Anjum A, Peters H, et al. HMDB 5.0: the human metabolome database for 2022. *Nucleic Acids Res*. (2022) 50(D1): D622–31. doi: 10.1093/nar/gkab1062

34. Heintz-Buschart A, Wilmes P. Human gut microbiome: function matters. *Trends Microbiol*. (2018) 26(7):563–74. doi: 10.1016/j.tim.2017.11.002

35. Greenhalgh K, Meyer KM, Aagaard KM, Wilmes P. The human gut microbiome in health: establishment and resilience of microbiota over a lifetime. *Environ Microbiol*. (2016) 18(7):2103–16. doi: 10.1111/1462-2920.13318

36. Turner JR. Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol*. (2009) 9(11):799–809. doi: 10.1038/nri2653

37. Martens EC, Neumann M, Desai MS. Interactions of commensal and pathogenic microorganisms with the intestinal mucosal barrier. *Nat Rev Microbiol*. (2018) 16(8):457–70. doi: 10.1038/s41579-018-0036-x

38. Belkaid Y, Harrison OJ. Homeostatic immunity and the microbiota. *Immunity*. (2017) 46(4):562–76. doi: 10.1016/j.immuni.2017.04.008

39. Evans JM, Morris LS, Marchesi JR. The gut microbiome: the role of a virtual organ in the endocrinology of the host. *J Endocrinol*. (2013) 218(3):R37–47. doi: 10.1530/JOE-13-0131

40. Ohlsson C, Nigro G, Boneca IG, Bäckhed F, Sansonetti P, Sjögren K. Regulation of bone mass by the gut microbiota is dependent on NOD1 and NOD2 signaling. *Cell Immunol*. (2017) 317:55–8. doi: 10.1016/j.cellimm.2017.05.003

41. Sjögren K, Engdahl C, Henning P, Lerner UH, Tremaroli V, Lagerquist MK, et al. The gut microbiota regulates bone mass in mice. *J Bone Miner Res*. (2012) 27 (6):1357–67. doi: 10.1002/jbmr.1588

42. Guss JD, Horsfield MW, Fontenele FF, Sandoval TN, Luna M, Apoorva F, et al. Alterations to the gut microbiome impair bone strength and tissue material properties. *J Bone Miner Res*. (2017) 32(6):1343–53. doi: 10.1002/jbmr.3114

43. Guss JD, Taylor E, Rouse Z, Roubert S, Higgins CH, Thomas CJ, et al. The microbial metagenome and bone tissue composition in mice with microbiome-induced reductions in bone strength. *Bone*. (2019) 127:146–54. doi: 10.1016/j.bone.2019.06.010

44. Ervin SM, Li H, Lim L, Roberts LR, Liang X, Mani S, et al. Gut microbial β-glucuronidases reactivate estrogens as components of the estrobolome that reactivate estrogens. *J Biol Chem*. (2019) 294(49):18586–99. doi: 10.1074/jbc.RA119.010950

45. Li JY, Chassaing B, Tyagi AM, Vaccaro C, Luo T, Adams J, et al. Sex steroid deficiency-associated bone loss is microbiota dependent and prevented by probiotics. *J Clin Invest*. (2016) 126(6):2049–63. doi: 10.1172/JCI86062

46. Yu M, Pal S, Paterson CW, Li J-Y, Tyagi AM, Adams J, et al. Ovariectomy induces bone loss via microbial-dependent trafficking of intestinal TNF+ T cells and Th17 cells. *J Clin Invest*. (2021) 131(4):e143137. doi: 10.1172/JCI143137

47. Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, et al. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell*. (2015) 161(2):264–76. doi: 10.1016/j.cell.2015.02.047

48. Ridlon JM, Kang D-J, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. *J Lipid Res*. (2006) 47(2):241–59. doi: 10.1194/jlr.R500013-JLR200

49. Yadav VK, Balaji S, Suresh PS, Liu XS, Lu X, Li Z, et al. Pharmacological inhibition of gut-derived serotonin synthesis is a potential bone anabolic treatment for osteoporosis. *Nat Med*. (2010) 16(3):308–12. doi: 10.1038/nm.2098

50. Ishizawa M, Akagi D, Makishima M. Lithocholic acid is a vitamin D receptor ligand that acts preferentially in the ileum. *Int J Mol Sci*. (2018) 19(7):1975. doi: 10.3390/ijms19071975

51. Brighton CA, Rievaj J, Kuhre RE, Glass LL, Schoonjans K, Holst JJ, et al. Bile acids trigger GLP-1 release predominantly by accessing basolaterally located G protein-coupled bile acid receptors. *Endocrinol*. (2015) 156(11):3961–70. doi: 10.1210/en.2015-1321

52. Wallace JL, Motta J-P, Buret AG. Hydrogen sulfide: an agent of stability at the microbiome-mucosa interface. *Am J Physiol Gastrointest Liver Physiol*. (2018) 314(2):G143–9. doi: 10.1152/ajpgi.00249.2017

53. Liu Y, Yang R, Liu X, Zhou Y, Qu C, Kikuiri T, et al. Hydrogen sulfide maintains mesenchymal stem cell function and bone homeostasis via regulation of Ca2+ channel sulfhydration. *Cell Stem Cell*. (2014) 15(1):66–78. doi: 10.1016/j.stem.2014.03.005

54. Grassi F, Tyagi AM, Calvert JW, Gambari L, Walker LD, Yu M, et al. Hydrogen sulfide is a novel regulator of bone formation implicated in the bone loss induced by estrogen deficiency. *J Bone Miner Res*. (2016) 31(5):949–63. doi: 10.1002/jbmr.2757

55. Li L, Rao S, Cheng Y, Zhuo X, Deng C, Xu N, et al. Microbial osteoporosis: the interplay between the gut microbiota and bones via host metabolism and immunity. *Microbiologyopen*. (2019) 8(8):e00810. doi: 10.1002/mbo3.810

56. Zaiss MM, Jones RM, Schett G, Pacifici R. The gut-bone axis: how bacterial metabolites bridge the distance. *J Clin Invest*. (2019) 129(8):3018–28. doi: 10.1172/JCI128521

57. Chen T-H, Chen W-M, Hsu K-H, Kuo C-D, Hung S-C. Sodium butyrate activates ERK to regulate differentiation of mesenchymal stem cells. *Biochem Biophys Res Commun*. (2007) 355(4):913–8. doi: 10.1016/j.bbrc.2007.02.057

58. Lucas S, Omata Y, Hofmann J, Böttcher M, Iljazovic A, Sarter K, et al. Short-chain fatty acids regulate systemic bone mass and protect from pathological bone loss. *Nat Commun*. (2018) 9(1):55. doi: 10.1038/s41467-017-02490-4

59. Yan J, Herzog Jeremy W, Tsang K, Brennan Caitlin A, Bower Maureen A, Garrett Wendy S, et al. Gut microbiota induce IGF-1 and promote bone formation and growth. *Proc Natl Acad Sci USA*. (2016) 113(47):E7554–63. doi: 10.1073/pnas.1607235113

60. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-y M, et al. The microbial metabolites, short-chain fatty acids, regulate colonic treg cell homeostasis. *Science*. (2013) 341(6145):569–73. doi: 10.1126/science.1241165

61. Fischer L, Herkner C, Kitte R, Dohnke S, Riewaldt J, Kretschmer K, et al. Foxp3(+) regulatory T cells in bone and hematopoietic homeostasis. *Front Endocrinol*. (2019) 10:578. doi: 10.3389/fendo.2019.00578

62. Tyagi AM, Yu M, Darby TM, Vaccaro C, Li JY, Owens JA, et al. The microbial metabolite butyrate stimulates bone formation via T regulatory cell-mediated regulation of WNT10B expression. *Immunity*. (2018) 49 (6):1116–31.e7. doi: 10.1016/j.immuni.2018.10.013

63. Li JY, Yu M, Pal S, Tyagi AM, Dar H, Adams J, et al. Parathyroid hormone-dependent bone formation requires butyrate production by intestinal microbiota. *J Clin Invest*. (2020) 130(4):1767–81. doi: 10.1172/JCI133473

64. Collins FL, Rios-Arce ND, Schepper JD, Parameswaran N, McCabe LR, Britton RA, et al. The potential of probiotics as a therapy for osteoporosis. *Microbiol Spectr*. (2017) 5(4):5.4.20. doi: 10.1128/microbiolspec.BAD-0015-2016

65. Li P, Sundh D, Ji B, Lappa D, Ye L, Nielsen J, et al. Metabolic alterations in older women with low bone mineral density supplemented with lactobacillus reuteri. *JBMR Plus*. (2021) 5(4):e10478. doi: 10.1002/jbm4.10478

66. Whisner CM, Weaver CM. Prebiotics and bone. In: LR McCabe, N Parameswaran, editors. *Understanding the gut-bone signaling axis: mechanisms*

*and therapeutic implications*. Cham: Springer International Publishing (2017). p. 201–24.

67. Sanders ME, Merenstein DJ, Reid G, Gibson GR, Rastall RA. Probiotics and prebiotics in intestinal health and disease: from biology to the clinic. *Nat Rev Gastroenterol Hepatol*. (2019) 16(10):605–16. doi: 10.1038/s41575-019-0173-3

68. Olsen I, Yamazaki K. Can oral bacteria affect the microbiome of the gut? *J Oral Microbiol*. (2019) 11(1):1586422. doi: 10.1080/20002297.2019.1586422

69. Kitamoto S, Nagao-Kitamoto H, Hein R, Schmidt TM, Kamada N. The bacterial connection between the oral cavity and the gut diseases. *J Dent Res*. (2020) 99(9):1021–9. doi: 10.1177/0022034520924633

70. Kitamoto S, Kamada N. Periodontal connection with intestinal inflammation: microbiological and immunological mechanisms. *Periodontol*. (2022) 89(1):142–53. doi: 10.1111/prd.12424

71. Said HS, Suda W, Nakagome S, Chinen H, Oshima K, Kim S, et al. Dysbiosis of salivary microbiota in inflammatory bowel disease and its association with oral immunological biomarkers. *DNA Res*. (2014) 21(1):15–25. doi: 10.1093/dnares/dst037

72. Atarashi K, Suda W, Luo C, Kawaguchi T, Motoo I, Narushima S, et al. Ectopic colonization of oral bacteria in the intestine drives TH1 cell induction and inflammation. *Science*. (2017) 358(6361):359–65. doi: 10.1126/science.aan4526

73. Kitamoto S, Nagao-Kitamoto H, Jiao Y, Gillilland MG, Hayashi A, Imai J, et al. The intermucosal connection between the mouth and gut in commensal pathobiont-driven colitis. *Cell*. (2020) 182(2):447–62.e14. doi: 10.1016/j.cell.2020.05.048

74. Hathaway-Schrader JD, Carson MD, Gerasco JE, Warner AJ, Swanson BA, Aguirre JI, et al. Commensal gut bacterium critically regulates alveolar bone homeostasis. *Lab Invest*. (2022) 102(4):363–75. doi: 10.1038/s41374-021-00697-0

75. Kato T, Yamazaki K, Nakajima M, Date Y, Kikuchi J, Hase K, et al. Oral administration of porphyromonas gingivalis alters the gut microbiome and Serum metabolome. *mSphere*. (2018) 3(5):e00460–18. doi: 10.1128/mSphere.00460-18

76. Simas AM, Kramer CD, Weinberg EO, Genco CA. Oral infection with a periodontal pathogen alters oral and gut microbiomes. *Anaerobe*. (2021) 71:102399. doi: 10.1016/j.anaerobe.2021.102399

77. Kobayashi R, Ogawa Y, Hashizume-Takizawa T, Kurita-Ochiai T. Oral bacteria affect the gut microbiome and intestinal immunity. *Pathog Dis*. (2020) 78(3). doi: 10.1093/femspd/ftaa024

78. Jia L, Tu Y, Jia X, Du Q, Zheng X, Yuan Q, et al. Probiotics ameliorate alveolar bone loss by regulating gut microbiota. *Cell Prolif*. (2021) 54(7):e13075. doi: 10.1111/cpr.13075

79. Sato K, Yamazaki K, Kato T, Nakanishi Y, Tsuzuno T, Yokoji-Takeuchi M, et al. Obesity-related gut microbiota aggravates alveolar bone destruction in experimental periodontitis through elevation of uric acid. *mBio*. (2021) 12(3):e00771–21. doi: 10.1128/mBio.00771-21

80. Barbour A, Elebyary O, Fine N, Oveisi M, Glogauer M. Metabolites of the oral microbiome: important mediators of multikingdom interactions. *FEMS Microbiol Rev*. (2022) 46(1). doi: 10.1093/femsre/fuab039

81. Tsuchida SNTMRiPDbMS. *Mol*. (2022) 27(9).

82. Baima G, Iaderosa G, Citterio F, Grossi S, Romano F, Berta GN, et al. Salivary metabolomics for the diagnosis of periodontal diseases: a systematic review with methodological quality assessment. *Metabolomics*. (2021) 17(1):1. doi: 10.1007/s11306-020-01754-3

83. Baima G, Corana M, Iaderosa G, Romano F, Citterio F, Meoni G, et al. Metabolomics of gingival crevicular fluid to identify biomarkers for periodontitis: a systematic review with meta-analysis. *J Periodontal Res*. (2021) 56(4):633–45. doi: 10.1111/jre.12872

84. Liebsch C, Pitchika V, Pink C, Samietz S, Kastenmüller G, Artati A, et al. The saliva metabolome in association to oral health Status. *J Dent Res*. (2019) 98(6):642–51. doi: 10.1177/0022034519842853

85. Sakanaka A, Kuboniwa M, Hashino E, Bamba T, Fukusaki E, Amano A. Distinct signatures of dental plaque metabolic byproducts dictated by periodontal inflammatory status. *Sci Rep*. (2017) 7(1):42818. doi: 10.1038/srep42818

86. Pei J, Li F, Xie Y, Liu J, Yu T, Feng X. Microbial and metabolomic analysis of gingival crevicular fluid in general chronic periodontitis patients: lessons for a predictive, preventive, and personalized medical approach. *EPMA Journal*. (2020) 11(2):197–215. doi: 10.1007/s13167-020-00202-5

87. Barnes VM, Kennedy AD, Panagakos F, Devizio W, Trivedi HM, Jönsson T, et al. Global metabolomic analysis of human Saliva and plasma from healthy and diabetic subjects, with and without periodontal disease. *PLoS One*. (2014) 9(8):e105181. doi: 10.1371/journal.pone.0105181

88. Lohinai Z, Keremi B, Szoko E, Tabi T, Szabo C, Tulassay Z, et al. Bacterial lysine decarboxylase influences human dental biofilm lysine content, biofilm accumulation, and subclinical gingival inflammation. *J Periodontol*. (2012) 83(8):1048–56. doi: 10.1902/jop.2011.110474

89. Yamada M, Takahashi N, Matsuda Y, Sato K, Yokoji M, Sulijaya B, et al. A bacterial metabolite ameliorates periodontal pathogen-induced gingival epithelial barrier disruption via GPR40 signaling. *Sci Rep*. (2018) 8(1):9008. doi: 10.1038/s41598-018-27408-y

90. Cueno ME, Ochiai K. Re-discovering periodontal butyric acid: new insights on an old metabolite. *Microb Pathog*. (2016) 94:48–53. doi: 10.1016/j.micpath.2015.10.006

91. Guan X, Li W, Meng H. A double-edged sword: role of butyrate in the oral cavity and the gut. *Mol Oral Microbiol*. (2021) 36(2):121–31. doi: 10.1111/omi.12322

92. Kurita-Ochiai T, Fukushima K, Ochiai K. Volatile fatty acids, metabolic by-products of periodontopathic bacteria, inhibit lymphocyte proliferation and cytokine production. *J Dent Res*. (1995) 74(7):1367–73. doi: 10.1177/00220345950740070801

93. Pöllänen MT, Overman DO, Salonen JI. Bacterial metabolites sodium butyrate and propionate inhibit epithelial cell growth in vitro. *J Periodontal Res*. (1997) 32(3):326–34. doi: 10.1111/j.1600-0765.1997.tb00541.x

94. Niederman R, Buyle-Bodin Y, Lu BY, Robinson P, Naleway C. Short-chain carboxylic acid concentration in human gingival crevicular fluid. *J Dent Res*. (1997) 76(1):575–9. doi: 10.1177/00220345970760010801

95. Lu R, Meng H, Gao X, Xu L, Feng X. Effect of non-surgical periodontal treatment on short chain fatty acid levels in gingival crevicular fluid of patients with generalized aggressive periodontitis. *J Periodontal Res*. (2014) 49(5):574–83. doi: 10.1111/jre.12137

96. Dallas R, Leonard B, Wali A, Bigler R, Sun W, Bultman Scott J. The warburg effect dictates the mechanism of butyrate-mediated histone acetylation and cell proliferation. *Mol Cell*. (2012) 48(4):612–26. doi: 10.1016/j.molcel.2012.08.033

97. Liu J, Wang Y, Meng H, Yu J, Lu H, Li W, et al. Butyrate rather than LPS subverts gingival epithelial homeostasis by downregulation of intercellular junctions and triggering pyroptosis. *J Clin Periodontol*. (2019) 46(9):894–907. doi: 10.1111/jcpe.13162

98. Magrin GL, Strauss FJ, Benfatti CA, Maia LC, Gruber R. Effects of short-chain fatty acids on human oral epithelial cells and the potential impact on periodontal disease: a systematic review of in vitro studies. *Int J Mol Sci*. (2020) 21(14):4895. doi: 10.3390/ijms21144895

99. Corrêa RO, Vieira A, Sernaglia EM, Lancellotti M, Vieira AT, Avila-Campos MJ, et al. Bacterial short-chain fatty acid metabolites modulate the inflammatory response against infectious bacteria. *Cell Microbiol*. (2017) 19(7):e12720. doi: 10.1111/cmi.12720

100. Montalvany-Antonucci CC, Duffles LF, de Arruda JAA, Zicker MC, de Oliveira S, Macari S, et al. Short-chain fatty acids and FFAR2 as suppressors of bone resorption. *Bone*. (2019) 125:112–21. doi: 10.1016/j.bone.2019.05.016

101. Overmyer KA, Rhoads TW, Merrill AE, Ye Z, Westphall MS, Acharya A, et al. Proteomics, lipidomics, metabolomics, and 16S DNA sequencing of dental plaque from patients with diabetes and periodontal disease. *Mol Cell Proteomics*. (2021) 20:100126. doi: 10.1016/j.mcpro.2021.100126

102. Califf KJ, Schwarzberg-Lipson K, Garg N, Gibbons Sean M, Caporaso JG, Slots J, et al. Multi-omics analysis of periodontal pocket microbial communities pre- and posttreatment. *mSystems*. (2017) 2(3):e00016–17. doi: 10.1128/mSystems.00016-17

103. Huang S, He T, Yue F, Xu X, Wang L, Zhu P, et al. Longitudinal multi-omics and microbiome meta-analysis identify an asymptomatic gingival state that links gingivitis, periodontitis, and aging. *mBio*. (2021) 12(2):e03281–20. doi: 10.1128/mBio.03281-20

frontiers | Frontiers in Dental Medicine

TYPE Mini Review
PUBLISHED 01 February 2023
DOI 10.3389/fdmed.2023.1129371



OPEN ACCESS

EDITED BY
Paulo Sergio Cerri,
Universidade Estadual Paulista, Brazil

REVIEWED BY
Vincent Everts,
VU Amsterdam, Netherlands

*CORRESPONDENCE
Thomas E. Van Dyke
✉ tvandyke@forsyth.org

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Systems Integration, a section of the journal Frontiers in Dental Medicine

RECEIVED 21 December 2022
ACCEPTED 09 January 2023
PUBLISHED 01 February 2023

CITATION
Abdalla HB and Van Dyke TE (2023) The impact of the soluble epoxide hydrolase cascade on periodontal tissues.
Front. Dent. Med 4:1129371.
doi: 10.3389/fdmed.2023.1129371



# The impact of the soluble epoxide hydrolase cascade on periodontal tissues

Henrique Ballassini Abdalla[1†] and Thomas E. Van Dyke[2,3*†]

[1]Faculdade São Leopoldo Mandic, Instituto de Pesquisa São Leopoldo Mandic, Campinas, Brazil, [2]Clinical and Translational Research, The Forsyth Institute, Cambridge, MA, United States, [3]Faculty of Medicine, Harvard University, Boston, MA, United States

Periodontitis is a chronic inflammatory disease with complex pathogenesis. Uncontrolled inflammation is driven by the immune system in response to accumulation of oral biofilm that leads to alveolar bone loss, bleeding, increased periodontal probing depth with loss of attachment of the connective tissues to the tooth, and ultimately, tooth loss. Soluble epoxide hydrolase (sEH) is an enzyme that converts epoxy fatty acids (EpFAs) produced by cytochrome P450 (CYP450) to an inactive diol. It has been shown that EpFAs display important features to counteract an exaggerated inflammatory process. Based upon this observation, inhibitors of sEH have been developed and are being proposed as a strategy to regulate proinflammatory inflammatory lipid mediator production and the chronicity of inflammation. This mini review focuses on the impact of sEH inhibition on periodontal tissues focusing on the mechanisms involved. The interaction between Specialized Pro-Resolving Mediators and sEH inhibition emerges as a significant mechanism of action of sEH inhibitors that was not formerly appreciated and provides new insight into the role SPMs may play in prevention and treatment of periodontitis.

KEYWORDS

periodontitis, inflammation, lipid mediator, soluble epoxide hydrolase (sEH), soluble epoxide hydrolase (sEH) inhibitors

## Introduction

Periodontitis is a chronic inflammatory disease with a complex pathogenesis, which encompasses the host immune system and oral microbiome dysbiosis (1–3). The uncontrolled inflammation in the periodontium leads to the destruction of hard and soft tissues and, eventually, tooth loss (4). The unwanted excessive inflammatory reaction in periodontitis is due to failure of endogenous inflammation resolution pathway activation (5). The cessation of the inflammatory process occurs when a balance between pro-inflammatory and pro-resolution mediators is achieved that determines health or disease (6, 7).

Inflammation is a natural and physiological reaction to injury or infection in all biological systems. This biochemical response is finely orchestrated and well-organized to fight pathogens and to restore homeostasis. It is generally accepted as a vital process for our existence. In an ideal scenario, an inflammatory reaction is self-limiting, characterized by a local increase of protein mediators (cytokines, chemokines) and lipid mediators (LMs) (e.g., prostaglandins and leukotrienes), vascular dilation and enhanced capillary permeability, and leukocyte trafficking and activation (8). The initiation or resolution of inflammation is dictated in large part by the metabolism of polyunsaturated fatty acids (PUFA) by cyclooxygenases (COX), lipoxygenases (LOX), or cytochrome P450 (CYP450) (9, 10).

Eicosanoids, a group of LMs, are oxidized derivates from the metabolism of arachidonic acid (ARA) by oxidative pathways, the COXs, LOXs, or CYP450 enzymes (8, 11). The resulting bioactive molecules, prostanoids, leukotrienes, hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EETs), and hydroperoxyeicosatetraenoic acids (HPETEs) are largely

generated in inflammation, with distinct biological functions (12). Although much is known about the metabolism of polyunsaturated fatty acids by the cyclooxygenases and lipoxygenases enzymatic pathways and the activities of their downstream metabolites (13), the cytochrome P450 pathways are less understood, and are the center of this mini review. Notably, the EETs, as well as epoxides of other long-chain polyunsaturated fatty acids (EpFA) generated by cytochrome P450 pathway, are important bioactive lipids with immunomodulatory actions in inflammation (14, 15). Most of these LMs are short-lived due to their rapid metabolization into inactive diols in the presence of soluble epoxide hydrolase (sEH), losing their ability to resolve inflammation (16). Worst, some of their diols contribute to inflammatory cytokine storm and block the initiation of the resolution phase (17). The sEH enzymes are largely found in liver, brain, spleen, kidney, intestine, and joints (18–20), and high sEH expression was detected in chronic osteolytic inflammatory disorders, such as periodontitis and arthritis (19, 21–23).

Here, this mini review dissects the mechanisms uncovered to date explaining how sEH inhibition impacts the inflammatory process in periodontal tissues, protects against alveolar bone resorption, and speculates possible interactions/synergism between metabolites derived from sEH inhibition and the resolvent lipid mediators (lipoxins, resolvins) in periodontal tissues.

## Periodontitis

Periodontitis is a chronic inflammatory and infectious disease culminating in a dysbiotic dental biofilm that disrupts the homeostasis of the subgingival environment (24). It is the sixth most prevalent disease among inflammatory osteolytic disorders worldwide, representing a significant public health problem (1, 2, 25). Clinically, periodontitis manifests as loss of clinical attachment, alveolar bone resorption, bleeding on probing, and periodontal pockets, and unlike gingivitis, these clinical symptoms are usually permanent. Individual periodontal susceptibility encompasses genetic, behavioral, and environmental factors that regulate the host immune response and generate ideal conditions for pathogenic biofilm microbial colonization (24, 26). Although microbial pathogens are associated with disease progression and severity, the molecular and biological basis of periodontitis is now realized to be the result of an excessive and uncontrolled inflammatory response rather than a classic infection with an exogenous organism(s) (7, 27). This shift in the periodontal disease paradigm began when increased levels of prostaglandin $E_2$ ($PGE_2$) were found in crevicular fluid of children and adults, and the levels of $PGE_2$ were correlated with disease severity. What caught the researcher's attention was that children had higher levels of $PGE_2$ than adults, and the capacity of $PGE_2$ to provoke periodontal tissue destruction (28–30). In this sense, the inflammation is an essential component of periodontal disease genesis; the tissues are destroyed by the host, not the bacteria.

As a chronic inflammatory disease, periodontitis stimulates a wide range of immune cells, from residents to infiltrating and patrolling cells, that disrupt tissue homeostasis and is characterized by a change in the immune cell composition (31). Additionally, the communication between the osseous and immune systems are intimately interconnected and responsible for bone destruction or remodeling (32–34). Alvarez and colleagues elegantly describe the spatiotemporal profile of the main gingival immune cell composition in ligature induced experimental periodontitis (35). Initially, neutrophils ($CD45^+LY6G^{high}LY6C^{mid}CD11b^{+/-}$) are the most abundant leukocyte cell in the gingiva, reaching their peak 24 h after ligature placement, indicating the activation of the innate immune response. This intense infiltration is accompanied by an over-expression of inflammatory cytokines (IL-1β, IL-6, IL-8, IL-12, and TNF-α), giving birth to a hyper-inflammatory phenomenon (36, 37). The transition between innate immunity to adaptive immune response begins on day 3 when tissue-resident macrophages are expanded, and circulating monocytes are recruited to be differentiated into M1-like macrophages ($CD45^+CD64^+CD11b^+MHCII^+$) (35). Macrophages are highly plastic cells that can exhibit dual roles in tissue repair or destruction, depending on their microenvironment (36, 38). Particularly, macrophage phenotypes, M1-like (pro-inflammatory subtype) or M2-like subsets (pro-resolving), are temporally associated with the different stages of experimental periodontitis progression (39). Although M1 macrophages are usually associated with an exacerbated inflammatory response, their presence and activation are needed to fight against pathogen invasion during the acute phase. They are implicated in producing several protein and lipid mediators (cytokines, chemokines, lipids mediators), which are fundamental to orchestrating the inflammatory response and guiding the return to tissue homeostasis, in a normal, self-limiting acute inflammatory response (40). On the other hand, resolving macrophages (M2-like) coordinate the resolution process of inflammation by removing dead cells through efferocytosis, producing anti-inflammatory cytokines (e.g., IL-10, IL-4, and TGF-β), counteracting osteoclast activity and boosting osteoblastic functions by augmented cystatin C (39, 40). Moreover, resolving macrophages are well-known synthesizers of Specialized Pro-resolving Mediators (SPMs), a fundamental lipid mediator class switching that defines inflammation termination and resolution stimulation (9, 41).

Failure of the acute response to resolve normally leads to chronicity and chronic inflammatory disease, which include periodontal disease. In experimental periodontitis, T cells ($CD45^+CD3^+$) represent roughly 70% of all cell populations in the gingiva, reaching the peak at day 10 post-ligature (35). Specifically, alveolar bone resorption relies on the imbalance between T-helper type 17 and regulatory T cells (Treg) (40, 42). Although Th17 cells have a physiological immune-protective role in the oral mucosa, their exaggerated activation establishes an interaction with the osteoclast by directly inducing RANKL expression by osteoblasts and periodontal ligament fibroblasts through IL-17A and IL-17F synthesis, with ultimately bone loss (34). The $CD4^+$ Th17 cells were first described in the early 2000s (43–45). This abnormal reaction is associated with augmented IL-23 levels, from the IL-12 cytokine family (46). Further, transforming growth factor-beta (TGF-beta) primes IL-23R, enhancing the Th17 responsiveness to IL-23 (45), culminating in intense neutrophil transmigration to inflamed sites and RANK/RANKL axis incitement (47). To the contrary, another subset of T cells, Tregs, are regulators of exaggerated inflammatory reactions, maintaining humoral tolerance and reestablishing homeostasis (48). The mainly immunosuppressive Treg features are

linked with the release of inhibitory cytokines, such as IL-10, TGF-beta (48), and IL-35 (49), and by dampening dendritic cells *via* the interaction between cytotoxic T-lymphocyte antigen 4 (CTLA4) and cluster of differentiation (CD) 80/86 (48). Curiously, in experimental periodontitis, Tregs from cervical lymph nodes lose their capacity to counteract osteoclastogenic activity, presenting lower expression of Foxp3, and show a Th17-type response (increased IL-17 gene expression) without fully transdifferentiating into Th17-like cells (50).

Endorsing the immunological aspects of periodontal disease progression, inflammatory lipid mediators are dramatically elevated in periodontal tissues and crevicular fluid, such as leukotriene $B_4$ ($LTB_4$) and prostaglandin $E_2$ ($PGE_2$) (28–30). Apart from inflammatory lipid mediators, differences in the Specialized Pro-Resolving Mediators (SPMs) and other lipids mediator profiles are associated with the stages of periodontal inflammation (51, 52). Gingival samples from healthy and periodontitis subjects showed distinct lipid profiles in PCA (Principal Component Analysis) of metabolipidomics (51). Notably, none of the SPMs are found to be higher in periodontitis than in healthy subjects, albeit several pathway markers for omega-6 driven SPMs (e.g., 5-HETE and 15-HETE), D-series resolvins (e.g., 4-HDHA and 7-HDHA), and E-series resolvins [15(S)-HEPE] were higher in periodontitis. Moreover, the resolvin E1 receptor (BLT1) was lower in periodontitis than in healthy subjects' samples (51). These findings suggest that in periodontitis, there is an effort by the body to re-establish homeostasis and initiate the resolution process through SPM synthesis; however, even though essential pathways seem to be activated, none of the final SPM metabolites were found at physiological levels, enough to exert cell function, and SPM receptor expression was decreased.

## Soluble epoxy hydrolase and its inhibition

John Casida's group led the discovery of soluble epoxide hydrolase in the 1970s, when they described an unknown epoxide hydrolase activity in the soluble fraction of liver homogenates (53–55). Interestingly, the fundamental biological role of sEH is proved by its conservation among species, from chordates to mammalians' (56), and it is mostly expressed in the liver, kidney, intestine, brain, and endothelial cells (57).

Soluble epoxide hydrolase was found to be essential for the hydrolysis of the epoxy fatty acids. The epoxy fatty acids are generated by polyunsaturated fatty acid metabolism [including ARA, linoleic acid (LA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA)] through the enzymatic activity of cytochrome P450, resulting in lipid mediators with a broad spectrum of biological functions at the systemic and cellular levels (58). The epoxidized metabolites are primarily anti-inflammatory and resolution lipids mediators, such as epoxyeicosatrienoic acids (EETs) from omega-6 ARA, epoxyeicosatrienoic acids (EEQs) from omega-3 EPA, and epoxydocosapentaenoic acids (EDPs) from omega-3 DHA (Wagner et al., 2017). However, in the presence of sEH (their principal regulatory enzyme), these epoxy metabolites are rapidly transformed into inactive diols, which could also possess pro-inflammatory functions (59).

In this regard, targeted inhibition of sEH during the inflammatory process, and consequently, enhancement of epoxy fatty acids bioavailability, offers an attractive strategy for inflammation control. The first inhibitors designed were too unstable for *in vivo* experiments (60). With the advent of crystallographic studies and the discovery of dicyclohexyl urea as a reversible inhibitor of soluble epoxide hydrolase (61), the next generation of inhibitors was produced with higher efficacy, stability, pharmacokinetics, and minor off-target activity (62). Since then, many studies have been carried out in several inflammatory models with promising results. Below, we summarize the findings on soluble epoxide hydrolase inhibition in periodontal tissues.

## Inhibition of soluble epoxy hydrolase in periodontal tissues and *in vitro* assays

The pharmacological inhibition of soluble epoxide hydrolase and its impact on inflammatory, autoimmune, and pain disorders has been widely explored (63–66). Nevertheless, its application in periodontitis or other orofacial conditions is new (18, 21–23, 67). There are only a few studies involving the inhibition of the soluble epoxide hydrolase enzyme in periodontal disease (21–23); therefore, we will address them in detail. Still, in our bibliographic search, we found only one article that shows the impact of EETs on osteoclasts (68) and another on fibroblasts (69), although both are not focused on oral tissues.

Trindade-da-Silva and colleagues initially demonstrated the protective effect of soluble epoxide hydrolase inhibitors (TPPU) on alveolar bone resorption in experimental periodontitis induced by *Aggregatibacter actinomycetemcomitans* (*Aa*), as exemplified in **Figure 1** in a ligature-induced periodontitis model (21). The potential bacteriostatic effect of the sEH inhibitor was discarded when no changes in *Aa*' growth was found in the presence of TPPU. Subsequently, by measuring the distance between the cemento–enamel junction and the alveolar bone crest, the researchers showed that by inhibiting soluble epoxide hydrolase, lower bone loss in infected animals was detected, altering the phenotype of experimental periodontitis. Interestingly, treatment with EETs, one of the CYP450 metabolite branches that is inactivated by sEH, did not prevented bone loss. Additionally, the treatment with sEH and EETs concomitantly, did not result in a greater prevention of bone loss. Corroborating, evidence was provided by genetic inhibition of soluble epoxide hydrolase by gene KO showing reduced bone loss, recapitulating the previous observations from the pharmacological inhibition by TPPU (21). Mechanistically, pharmacological inhibition and genetic ablation decreased activation of the RANK/RANKL/OPG axis in gingival tissue. In agreement, the reduced protein expression of MCP-1 (monocyte chemotactic protein 1), a vital monocyte recruiter associated with lower levels of F4/80 (EGF-like module-containing mucin-like hormone receptor-like 1) in the gingiva, endorses that the protective effect of sEH inhibition is related to the regulation of the exaggerated inflammation and the immune system response (21). The decreased inflammatory process was tracked by two essential downstream stress kinases, mitogen-activated protein kinase phosphorylation (p38 and JNK 1/2), which ultimately lead to nuclear factor kappa B (NFκB) activation (70). Animals treated with TPPU, TPPU and EETs, or in sEH KOs, showed greatly

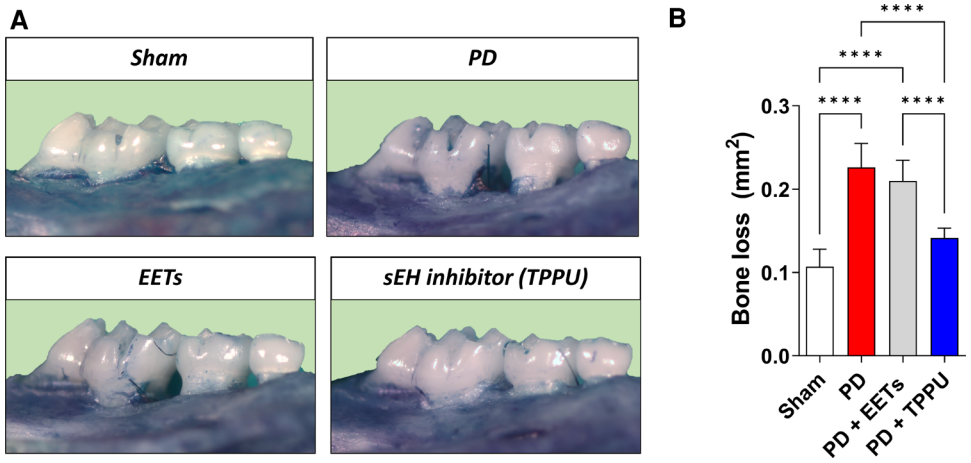



FIGURE 1
Inhibition of soluble epoxide hydrolase prevents alveolar bone loss in experimental periodontitis in mice. (A) Representative images from a palatal view of maxillary molars. TPPU was used as the soluble epoxide hydrolase inhibitor. (B) Bone loss was quantified as the area between the cementum-enamel junction and the alveolar bone. PD, periodontal disease; EETs, epoxyeicosatrienoic acids. ****$P < 0.0001$. The data are expressed as mean ± S.D; $n = 5$ animals per group.

reduced phosphorylation of p38 and JNK 1/2. Finally, pharmacological sEH inhibition and knockout animals ($sEH^{-/-}$) showed inhibition of phosphorylation of the ER stress sensor (PERK, protein kinase RNA-like ER kinase); eIF2α, eukaryotic initiation factor 2α; IRE1, inositol-requiring enzyme 1; sXBP1, spliced X-box binding protein 1 and associated apoptosis (c-Caspase-3 and immunoglobulin binding protein) (21).

Napimoga and collaborators, in a succeeding study by the same research group, showed that inflamed gingival tissue induced by experimental periodontitis expressed higher levels of sEH than control animals. Pharmacological inhibition of sEH dampened this expression, and correlated with lessening disease severity (22). Using an RNA array to explore the innate and adaptive immune systems, sEH inhibition diminished the expression of toll-like receptors 1 and 9 (Trl1 and Trl9), which play a crucial role in inflammatory cytokine release upon triacylated lipopeptide recognition (71) and activation of osteoclastic functions (72). T cells were also affected. The expression of Cd8 and Cd4 was diminished, as well as Cd40l, interferon-alpha2 (Ifnα2), and interferon-beta (Ifnβ) (21). Downregulation of Cd40l impairs B-cell activation and, therefore, the production of IL-2, IL-6, and TNF-alpha (73). The signal transducer and activator of transcription 4 (Stat4) is a factor that contributes to IL-12, IL-23, and IFN-1 production, in addition to differentiating Th1 and Th17 cells (74), which was also reduced by sEH inhibition. These findings reinforce the concept that by inhibiting sEH, the unwanted lymphocyte response is managed, as also demonstrated in a collagen-induced model of arthritis (19), preventing osteoclastogenic activity in the periodontium (21) and knee joint (19).

Recently, Abdalla and coworkers thoroughly characterized the impact of the sEH/EET axis on gingival macrophage plasticity in experimental periodontitis in mice. The work revealed for the first time that pharmacological inhibition of sEH fosters communication between epoxy fatty acid metabolites, increasing the levels of Specialized Pro-Resolving Mediators [e.g., resolvin (Rv) E-series and lipoxins] in saliva, as well as their respective receptors in the gingival tissues (23).

Mechanistically, pharmacological sEH inhibition suppressed alveolar bone loss *via* actions on inflammatory osteolytic factors, such as Il17a and RANKL. In metabolipidomic analyses, soluble epoxide hydrolase inhibitor treated animals showed lipid profiles that were distinct from experimental periodontitis and control animals in two-dimensional and three-dimensional Principal Component Analyses. The foremost lipid mediators enhanced by sEH inhibition were RvE1, RvE2, and $LXA_4$, well-known SPMs with robust immunoresolvent features that guide healing. Moreover, 20-hydroxy $LTB_4$ was enhanced, inferring an inactivation of $LTB_4$, a critical inflammatory lipid mediator. Further, the Specialized Pro-Resolving Mediator receptors (LTB4R1, CMKLR1/ChemR23, and ALX/FPR2) were also found to increase in gingival tissue, suggesting greater effectiveness SPM activity at the site of inflammation. In macrophages, the pharmacological inhibition of soluble epoxide hydrolase stimulated a dynamic transcriptional reprogramming of inflammatory macrophages toward resolving macrophages (characterized by $CD11c^+/CD206^+$ double-positive cells in the $CD45^+/CD11b^+/CD64^+$ macrophage population), associated with reduced expression of Il1β, TNFα, Il12, and Nos2. Finally, *in vitro* assay revealed that sEH inhibition and EET treatment triggered SPM release in bone marrow derived macrophages (BMDMs) in both inflammatory and resolvents macrophages (23). These findings are summarized in **Figure 2**.

The direct influence of the EETs/sEH/DHET axis on osteoclast differentiation and activity was explored *in vitro* using BMMCs (Bone marrow mononuclear cells) and RAW264.7 murine cells (68). Authors showed that DHETs, the inactive diol form of EETs, could not reduce TRAP-positive cells, but increased their number. Differently, treatment with EETs or sEH inhibitor (TPPU) significantly diminished the number of multinucleated TRAP-positive cells. Likewise, bone resorption pits were hardly impaired by EETs and sEH inhibition, as well as expression of RANK, TRAP, cathepsin K (CK), and matrix metalloproteinase (MMP)-9. Further, in an osteoblast precursor cell line (MC3T3), EETs reduced the ratio between RANKL:OPG (68). In TGF-β1-induced activation of murine

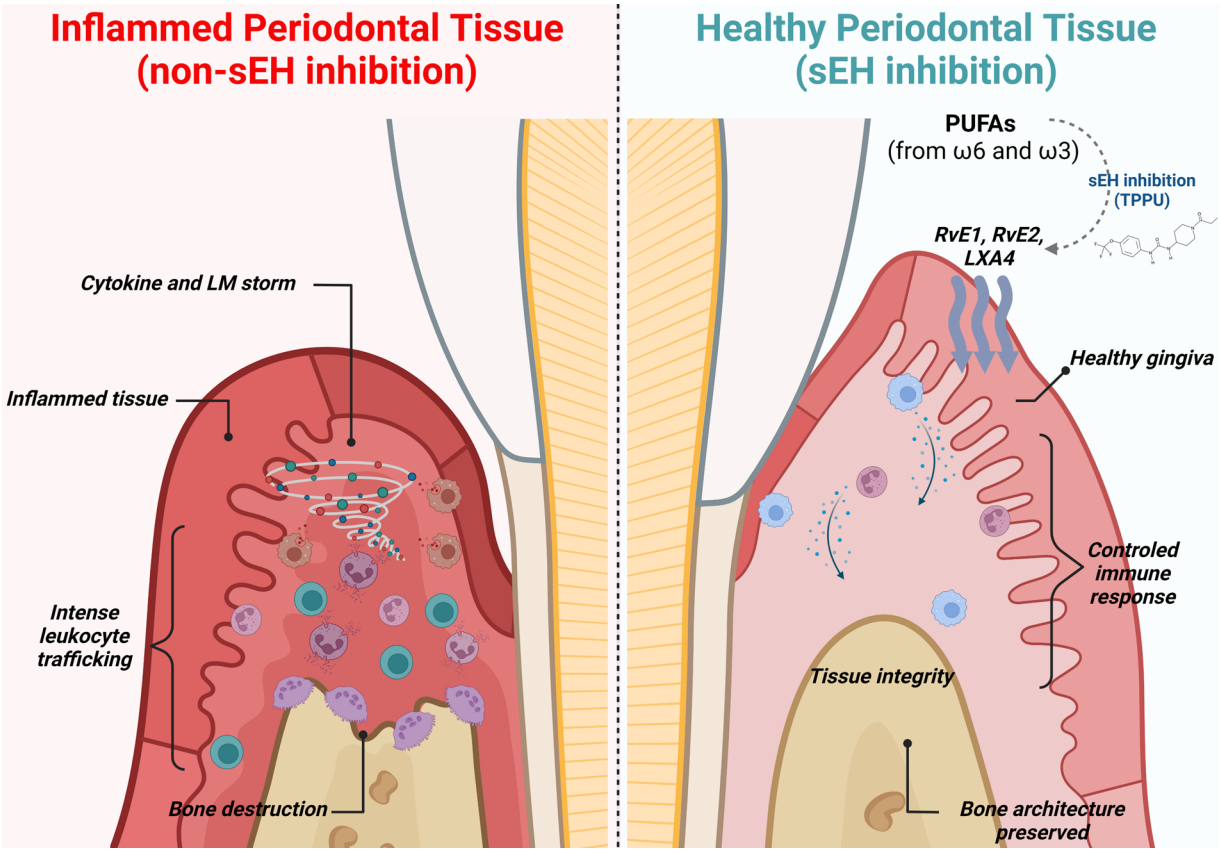



FIGURE 2
Immune modulation and lipid mediator synthesis induced by sEH inhibition in experimental periodontitis. During periodontitis, the immune system drives an unwanted and uncontrolled inflammatory reaction, leading to an intense release of inflammatory cytokines, chemokines, and lipid mediators, ultimately leading to alveolar bone loss, gingival tissue damage, and increased probing depth (left panel). Pharmacological inhibition of sEH improves the bioavailability of epoxy fatty acids (EpFAs), shifting polyunsaturated fatty acid (PUFA) metabolism and favoring production of Specialized Pro-Resolving Mediators. Further, macrophages undergo phenotypic reprogramming towards resolving and repairing features associated with releasing anti-inflammatory cytokines. Innate immunity is controlled and well-orchestrated. Finally, inhibition of sEH prevents osteoclastic activation, preventing alveolar bone loss.

fibroblasts (NIH3T3), EETs attenuate cell activation by impairing the expression of collagen, smooth muscle alpha-actin (α-SMA), and proliferating cell nuclear antigen (PCNA) in a peroxisome proliferation activated receptor γ (PPARγ) dependent-manner (69).

## Conclusions and perspective

The pharmacological inhibition of sEH has shown impressive results in inflammatory diseases and has been the subject of extensive research. Concerning the dental medicine area, including painful orofacial conditions and periodontal disease, a few studies have been conducted, revealing promising findings. As a note, sEH inhibitors are in the clinical development phase, making them a promising forthcoming therapeutic strategy. Nevertheless, a profound molecular mechanistic analysis of how sEH inhibition acts through the immune system must be carried out. Future research should deeply analyze the impact of sEH inhibition on immune system cells and how they respond in its absence. Nevertheless, recent findings demonstrate that the inhibition of sEH influences the production of SPMs (omega-3 and -6 fatty acids metabolites from CYP450), which paves the way for a new perspective on its mechanism of action, as well as pharmacological implications, as they boost resolution pathways of inflammation rather than silencing them.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

## Funding

This work was supported in part by USPHS grants DE025020.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## References

1. Hajishengallis G, Chavakis T. Local and systemic mechanisms linking periodontal disease and inflammatory comorbidities. *Nat Rev Immunol*. (2021) 21(7):426–40. doi: 10.1038/s41577-020-00488-6

2. Lamont RJ, Hajishengallis G. Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends Mol Med*. (2015) 21(3):172–83. doi: 10.1016/j.molmed.2014.11.004

3. Alvarez C, Monasterio G, Cavalla F, Córdova LA, Hernández M, Heymann D, et al. Osteoimmunology of oral and maxillofacial diseases: translational applications based on biological mechanisms. *Front Immunol*. (2019) 10:1664. doi: 10.3389/fimmu.2019.01664

4. Van Dyke TE, Sima C. Understanding resolution of inflammation in periodontal diseases: is chronic inflammatory periodontitis a failure to resolve? *Periodontol 2000*. (2020) 82(1):205–13. doi: 10.1111/prd.12317

5. Van Dyke TE. Proresolving lipid mediators: potential for prevention and treatment of periodontitis. *J Clin Periodontol*. (2011) 38(Suppl 11):119–25. doi: 10.1111/j.1600-051X.2010.01662.x

6. Balta MG, Papathanasiou E, Blix IJ, Van Dyke TE. Host modulation and treatment of periodontal disease. *J Dent Res*. (2021) 100(8):798–809. doi: 10.1177/0022034521995157

7. Van Dyke TE. Shifting the paradigm from inhibitors of inflammation to resolvers of inflammation in periodontitis. *J Periodontol*. (2020) 91(Suppl 1):S19–25. doi: 10.1002/JPER.20-0088

8. Serhan CN. Pro-resolving lipid mediators are leads for resolution physiology. *Nature*. (2014) 510(7503):92–101. doi: 10.1038/nature13479

9. Panigrahy D, Gilligan MM, Serhan CN, Kashfi K. Resolution of inflammation: an organizing principle in biology and medicine. *Pharmacol Ther*. (2021) 227:107879. doi: 10.1016/j.pharmthera.2021.107879

10. Maddipati KR. Non-inflammatory physiology of "inflammatory" mediators - unalamation, a new paradigm. *Front Immunol*. (2020) 11:580117. doi: 10.3389/fimmu.2020.580117

11. Wang D, Dubois RN. Eicosanoids and cancer. *Nat Rev Cancer*. (2010) 10(3):181–93. doi: 10.1038/nrc2809

12. Haeggström JZ, Funk CD. Lipoxygenase and leukotriene pathways: biochemistry, biology, and roles in disease. *Chem Rev*. (2011) 111(10):5866–98. doi: 10.1021/cr200246d

13. Hoxha M, Zappacosta B. CYP-derived eicosanoids: implications for rheumatoid arthritis. *Prostaglandins Other Lipid Mediat*. (2020) 146:106405. doi: 10.1016/j.prostaglandins.2019.106405

14. Zeldin DC. Epoxygenase pathways of arachidonic acid metabolism. *J Biol Chem*. (2001) 276(39):36059–62. doi: 10.1074/jbc.R100030200

15. Hammock BD, Wang W, Gilligan MM, Panigrahy D. Eicosanoids: the overlooked storm in coronavirus disease 2019 (COVID-19)? *Am J Pathol*. (2020) 190(9):1782–8. doi: 10.1016/j.ajpath.2020.06.010

16. Wang B, Wu L, Chen J, Dong L, Chen C, Wen Z, et al. Metabolism pathways of arachidonic acids: mechanisms and potential therapeutic targets. *Signal Transduct Target Ther*. (2021) 6(1):94. doi: 10.1038/s41392-020-00443-w

17. Kodani SD, Hammock BD. The 2014 bernard B. Brodie award lecture-epoxide hydrolases: drug metabolism to therapeutics for chronic pain. *Drug Metab Dispos*. (2015) 43(5):788–802. doi: 10.1124/dmd.115.063339

18. Abdalla HB, Napimoga MH, Teixeira JM, Trindade-da-Silva CA, Pieroni VL, Dos Santos Araújo FSM, et al. Soluble epoxide hydrolase inhibition avoid formalin-induced inflammatory hyperalgesia in the temporomandibular joint. *Inflammopharmacology*. (2022) 30(3):981–90. doi: 10.1007/s10787-022-00965-5

19. Trindade-da-Silva CA, Clemente-Napimoga JT, Abdalla HB, Rosa SM, Ueira-Vieira C, Morisseau C, et al. Soluble epoxide hydrolase inhibitor, TPPU, increases regulatory T cells pathway in an arthritis model. *FASEB J*. (2020) 34(7):9074–86. doi: 10.1096/fj.202000415R

20. Norwood S, Liao J, Hammock BD, Yang GY. Epoxyeicosatrienoic acids and soluble epoxide hydrolase: potential therapeutic targets for inflammation and its induced carcinogenesis. *Am J Transl Res*. (2010) 2(4):447–57. PMID: 20733953; PMCID: PMC2923867

21. Trindade-da-Silva CA, Bettaieb A, Napimoga MH, Lee KSS, Inceoglu B, Ueira-Vieira C, et al. Soluble epoxide hydrolase pharmacological inhibition decreases alveolar bone loss by modulating host inflammatory response, RANK-related signaling, endoplasmic Reticulum stress, and apoptosis. *J Pharmacol Exp Ther*. (2017) 361(3):408–16. doi: 10.1124/jpet.116.238113

22. Napimoga MH, Rocha EP, Trindade-da-Silva CA, Demasi APD, Martinez EF, Macedo CG, et al. Soluble epoxide hydrolase inhibitor promotes immunomodulation to inhibit bone resorption. *J Periodontal Res*. (2018) 53(5):743–9. doi: 10.1111/jre.12559

23. Abdalla HB, Alvarez C, Wu YC, Rojas P, Hammock BD, Maddipati KR, et al. Soluble epoxide hydrolase inhibition enhances specialized pro-resolving lipid mediator production and promotes macrophage plasticity. *Br J Pharmacol*. (2022). doi: 10.1111/bph.16009. [Epub ahead of print]

24. Papapanou PN, Sanz M, Buduneli N, Dietrich T, Feres M, Fine DH, et al. Periodontitis: consensus report of workgroup 2 of the 2017 world workshop on the classification of periodontal and peri-implant diseases and conditions. *J Periodontol*. (2018) 89(Suppl 1):S173–82. doi: 10.1002/JPER.17-0721

25. Kassebaum NJ, Smith AGC, Bernabé E, Fleming TD, Reynolds AE, Vos T, et al. Global, regional, and national prevalence, incidence, and disability-adjusted life years for oral conditions for 195 countries, 1990-2015: a systematic analysis for the global burden of diseases, injuries, and risk factors. *J Dent Res*. (2017) 96(4):380–7. doi: 10.1177/0022034517693566

26. Kurgan S, Kantarci A. Molecular basis for immunohistochemical and inflammatory changes during progression of gingivitis to periodontitis. *Periodontol 2000*. (2018) 76(1):51–67. doi: 10.1111/prd.12146

27. Van Dyke TE, Bartold PM, Reynolds EC. The nexus between periodontal inflammation and dysbiosis. *Front Immunol*. (2020) 11:511. doi: 10.3389/fimmu.2020.00511

28. Offenbacher S, Odle BM, Gray RC, Van Dyke TE. Crevicular fluid prostaglandin E levels as a measure of the periodontal disease status of adult and juvenile periodontitis patients. *J Periodontal Res*. (1984) 19(1):1–13. doi: 10.1111/j.1600-0765.1984.tb01190.x

29. Offenbacher S, Odle BM, Van Dyke TE. The use of crevicular fluid prostaglandin E2 levels as a predictor of periodontal attachment loss. *J Periodontal Res*. (1986) 21(2):101–12. doi: 10.1111/j.1600-0765.1986.tb01443.x

30. Van Dyke TE, Lester MA, Shapira L. The role of the host response in periodontal disease progression: implications for future treatment strategies. *J Periodontol*. (1993) 64(Suppl 8S):792–806. doi: 10.1902/jop.1993.64.8s.792

31. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic inflammation. *Nat Rev Immunol*. (2015) 15(1):30–44. doi: 10.1038/nri3785

32. Arron JR, Choi Y. Bone versus immune system. *Nature*. (2000) 408(6812):535–6. doi: 10.1038/35046196

33. Takayanagi H, Ogasawara K, Hida S, Chiba T, Murata S, Sato K, et al. T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-gamma. *Nature*. (2000) 408(6812):600–5. doi: 10.1038/35046102

34. Ikeuchi T, Moutsopoulos NM. Osteoimmunology in periodontitis; a paradigm for Th17/IL-17 inflammatory bone loss. *Bone*. (2022) 163:116500. doi: 10.1016/j.bone.2022.116500

35. Alvarez C, Abdalla H, Sulliman S, Rojas P, Wu YC, Almarhoumi R, et al. Rve1 impacts the gingival inflammatory infiltrate by inhibiting the T cell response in experimental periodontitis. *Front Immunol*. (2021) 12:664756. doi: 10.3389/fimmu.2021.664756

36. Ling MR, Chapple IL, Matthews JB. Peripheral blood neutrophil cytokine hyper-reactivity in chronic periodontitis. *Innate Immun*. (2015) 21(7):714–25. doi: 10.1177/1753425915589387

37. Dutzan N, Konkel JE, Greenwell-Wild T, Moutsopoulos NM. Characterization of the human immune cell network at the gingival barrier. *Mucosal Immunol*. (2016) 9(5):1163–72. doi: 10.1038/mi.2015.136

38. Chazaud B. Macrophages: supportive cells for tissue repair and regeneration. *Immunobiology*. (2014) 219(3):172–8. doi: 10.1016/j.imbio.2013.09.001

39. Viniegra A, Goldberg H, Çil Ç, Fine N, Sheikh Z, Galli M, et al. Resolving macrophages counter osteolysis by anabolic actions on bone cells. *J Dent Res*. (2018) 97(10):1160–9. doi: 10.1177/0022034518777973

40. Garlet GP, Giannobile WV. Macrophages: the bridge between inflammation resolution and tissue repair? *J Dent Res*. (2018) 97(10):1079–81. doi: 10.1177/0022034518785857

41. Dalli J, Serhan C. Macrophage proresolving mediators-the when and where. *Microbiol Spectr*. (2016) 4(3):10.1128/microbiolspec.MCHD-0001-2014. doi: 10.1128/microbiolspec.MCHD-0001-2014

42. Dutzan N, Kajikawa T, Abusleme L, Greenwell-Wild T, Zuazo CE, Ikeuchi T, et al. A dysbiotic microbiome triggers TH17 cells to mediate oral mucosal immunopathology

in mice and humans. *Sci Transl Med*. (2018) 10(463):eaat0797. doi: 10.1126/scitranslmed.aat0797

43. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*. (2006) 126(6):1121–33. doi: 10.1016/j.cell.2006.07.035

44. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity*. (2006) 24(2):179–89. doi: 10.1016/j.immuni.2006.01.001

45. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature*. (2006) 441(7090):231–4. doi: 10.1038/nature04754

46. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity*. (2000) 13(5):715–25. doi: 10.1016/S1074-7613(00)00070-4

47. Pan W, Wang Q, Chen Q. The cytokine network involved in the host immune response to periodontitis. *Int J Oral Sci*. (2019) 11(3):30. doi: 10.1038/s41368-019-0064-z

48. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nat Rev Immunol*. (2008) 8(7):523–32. doi: 10.1038/nri2343

49. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*. (2007) 450(7169):566–9. doi: 10.1038/nature06306

50. Alvarez C, Suliman S, Almarhoumi R, Vega ME, Rojas C, Monasterio G, et al. Regulatory T cell phenotype and anti-osteoclastogenic function in experimental periodontitis. *Sci Rep*. (2020) 10(1):19018. doi: 10.1038/s41598-020-76038-w

51. Ferguson B, Bokka NR, Maddipati KR, Ayilavarapu S, Weltman R, Zhu L, et al. Distinct profiles of specialized pro-resolving lipid mediators and corresponding receptor gene expression in periodontal inflammation. *Front Immunol*. (2020) 11:1307. doi: 10.3389/fimmu.2020.01307

52. Elabdeen HR, Mustafa M, Szklenar M, Rühl R, Ali R, Bolstad AI. Ratio of pro-resolving and pro-inflammatory lipid mediator precursors as potential markers for aggressive periodontitis. *PLoS One*. (2013) 8(8):e70838. doi: 10.1371/journal.pone.0070838

53. Gill SS, Hammock BD, Yamamoto I, Casida JE. Preliminary chromatographic studies on the metabolites and photodecomposition products of the juvenoid 1-(4'-ethylphenoxy)-6,7-epoxy-3,7-dimethyl-2-octene. In: JJ Menn, M Beroza, editors. *Insect juvenile hormones: Chemistry and action*. New York: Academic Press (1972). p. 177–89.

54. Gill SS, Hammock BD, Casida JE. Mammalian metabolism and environmental degradation of the juvenoid 1-(4'-ethylphenoxy)-3,7-dimethyl-6,7-epoxy-trans-2-octene and related compounds. *J Agric Food Chem*. (1974) 22(3):386–95. doi: 10.1021/jf60193a058

55. Hammock BD, Gill SS, Stamoudis V, Gilbert LI. Soluble mammalian epoxide hydratase: action on juvenile hormone and other terpenoid epoxides. *Comp Biochem Physiol B*. (1976) 53(2):263–5. doi: 10.1016/0305-0491(76)90045-6

56. Harris TR, Hammock BD. Soluble epoxide hydrolase: gene structure, expression and deletion. *Gene*. (2013) 526(2):61–74. doi: 10.1016/j.gene.2013.05.008

57. Enayetallah AE, French RA, Thibodeau MS, Grant DF. Distribution of soluble epoxide hydrolase and of cytochrome P450 2C8, 2C9, and 2J2 in human tissues. *J Histochem Cytochem*. (2004) 52(4):447–54. doi: 10.1177/002215540405200403

58. Dyall SC, Balas L, Bazan NG, Brenna JT, Chiang N, da Costa Souza F, et al. Polyunsaturated fatty acids and fatty acid-derived lipid mediators: recent advances in the understanding of their biosynthesis, structures, and functions. *Prog Lipid Res*. (2022) 86:101165. doi: 10.1016/j.plipres.2022.101165

59. Spector AA. Arachidonic acid cytochrome P450 epoxygenase pathway. *J Lipid Res*. (2009) 50:S52–6. doi: 10.1194/jlr.R800038-JLR200

60. Mullin CA, Hammock BD. Chalcone oxides–potent selective inhibitors of cytosolic epoxide hydrolase. *Arch Biochem Biophys*. (1982) 216(2):423–39. doi: 10.1016/0003-9861(82)90231-4

61. Morisseau C, Goodrow MH, Dowdy D, Zheng J, Greene JF, Sanborn JR, et al. Potent urea and carbamate inhibitors of soluble epoxide hydrolases. *Proc Natl Acad Sci U S A*. (1999) 96(16):8849–54. doi: 10.1073/pnas.96.16.8849

62. Morisseau C, Hammock BD. Impact of soluble epoxide hydrolase and epoxyeicosanoids on human health. *Annu Rev Pharmacol Toxicol*. (2013) 53:37–58. doi: 10.1146/annurev-pharmtox-011112-140244

63. Wagner KM, McReynolds CB, Schmidt WK, Hammock BD. Soluble epoxide hydrolase as a therapeutic target for pain, inflammatory and neurodegenerative diseases. *Pharmacol Ther*. (2017) 180:62–76. doi: 10.1016/j.pharmthera.2017.06.006

64. Fishbein A, Hammock BD, Serhan CN, Panigrahy D. Carcinogenesis: failure of resolution of inflammation? *Pharmacol Ther*. (2021) 218:107670. doi: 10.1016/j.pharmthera.2020.107670

65. Wang Y, Wagner KM, Morisseau C, Hammock BD. Inhibition of the soluble epoxide hydrolase as an analgesic strategy: a review of preclinical evidence. *J Pain Res*. (2021) 14:61–72. doi: 10.2147/JPR.S241893

66. Shan J, Hashimoto K. Soluble epoxide hydrolase as a therapeutic target for neuropsychiatric disorders. *Int J Mol Sci*. (2022) 23(9):4951. doi: 10.3390/ijms23094951

67. Teixeira JM, Abdalla HB, Basting RT, Hammock BD, Napimoga MH, Clemente-Napimoga JT. Peripheral soluble epoxide hydrolase inhibition reduces hypernociception and inflammation in albumin-induced arthritis in temporomandibular joint of rats. *Int Immunopharmacol*. (2020) 87:106841. doi: 10.1016/j.intimp.2020.106841

68. Guan H, Zhao L, Cao H, Chen A, Xiao J. Epoxyeicosanoids suppress osteoclastogenesis and prevent ovariectomy-induced bone loss. *FASEB J*. (2015) 29(3):1092–101. doi: 10.1096/fj.14-262055

69. Tao JH, Liu T, Zhang CY, Zu C, Yang HH, Liu YB, et al. Epoxyeicosatrienoic acids inhibit the activation of murine fibroblasts by blocking the TGF-β1-Smad2/3 signaling in a PPARγ-dependent manner. *Oxid Med Cell Longev*. (2022) 2022:7265486. doi: 10.1155/2022/7265486

70. Nandipati KC, Subramanian S, Agrawal DK. Protein kinases: mechanisms and downstream targets in inflammation-mediated obesity and insulin resistance. *Mol Cell Biochem*. (2017) 426(1-2):27–45. doi: 10.1007/s11010-016-2878-8

71. Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik SG, et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. *Cell*. (2007) 130(6):1071–82. doi: 10.1016/j.cell.2007.09.008

72. Matsumoto C, Oda T, Yokoyama S, Tominari T, Hirata M, Miyaura C, et al. Toll-like receptor 2 heterodimers, TLR2/6 and TLR2/1 induce prostaglandin E production by osteoblasts, osteoclast formation and inflammatory periodontitis. *Biochem Biophys Res Commun*. (2012) 428(1):110–5. doi: 10.1016/j.bbrc.2012.10.016

73. Figueredo CM, Lira-Junior R, Love RM. T and B cells in periodontal disease: new functions in A complex scenario. *Int J Mol Sci*. (2019) 20(16):3949. doi: 10.3390/ijms20163949

74. Cope AP, Schulze-Koops H, Aringer M. The central role of T cells in rheumatoid arthritis. *Clin Exp Rheumatol*. (2007) 25(5 Suppl 46):S4–11. PMID: 17977483

frontiers | Frontiers in Dental Medicine

TYPE Original Research
PUBLISHED 10 March 2023
DOI 10.3389/fdmed.2023.1124084



OPEN ACCESS

EDITED BY
Yong-Hee Patricia Chun,
The University of Texas Health Science Center at San Antonio, United States

REVIEWED BY
Chad Novince,
Medical University of South Carolina, United States
Masaru Kaku,
Niigata University, Japan
Shuo Chen,
The University of Texas Health Science Center at San Antonio, United States

*CORRESPONDENCE
Karin Nagasaki
nagasaki.karin.r2@dc.tohoku.ac.jp

†These authors have contributed equally to this work and share senior authorship

SPECIALTY SECTION
This article was submitted to Systems Integration, a section of the journal Frontiers in Dental Medicine

RECEIVED 14 December 2022
ACCEPTED 23 February 2023
PUBLISHED 10 March 2023

CITATION
Nagasaki K, Nagasaki A, Taylor JM, Kear BD, Ma Y, Somerman MJ and Gavrilova O (2023) The RGD region of bone sialoprotein affects metabolic activity in mice.
Front. Dent. Med 4:1124084.
doi: 10.3389/fdmed.2023.1124084



# The RGD region of bone sialoprotein affects metabolic activity in mice

Karin Nagasaki[1,2]*, Atsuhiro Nagasaki[1,3], Jocelyn M. Taylor[1], Bernice D. Kear[1], Yinyan Ma[4], Martha J. Somerman[1†] and Oksana Gavrilova[4†]

[1]Laboratory of Oral Connective Tissue Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), National Institutes of Health (NIH), Bethesda, MD, United States, [2]Department of Periodontology and Endodontology, Tohoku University Graduate School of Dentistry, Sendai, Japan, [3]Division of Molecular and Regenerative Prosthodontics, Tohoku University Graduate School of Dentistry, Sendai, Japan, [4]Mouse Metabolism Core, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health (NIH), Bethesda, MD, United States

**Introduction:** Bone sialoprotein (BSP) is a key regulator of mineralized tissue formation. Previously, we generated BSP-KAE knock-in mice (KAEKI mice) by substituting a non-function KAE (lysine-alanine-glutamic acid) for the integrin-binding RGD (arginine-glycine-aspartic acid) sequence and reported a vital role of the BSP-RGD motif in modulating the periodontal ligament (PDL). Specifically, histologically a disorganization of the PDL was noted, resulting in a weakened function of the PDL as measured by dynamic mechanical analysis. Intriguingly, also noted was a weight gain as KAEKI mice aged. While several proteins associated with mineralized tissues are reported to affect energy metabolism, the metabolic role of the BSP-RGD region has yet to be elucidated. Here we focus on defining the role of the BSP-RGD region in metabolic activity.
**Methods:** Body weight, body composition, and caloric intake were measured in wild type (WT) and KAEKI mice. Energy expenditure was estimated using energy balance technique. Epididymal fat, interscapular fat, and liver were harvested for histological analysis. Systemic metabolic phenotype was assessed by sera analyses, insulin tolerance and glucose tolerance tests.
**Results:** The results showed that KAEKI mice developed mild obesity starting from 13 weeks postnatal (wpn). The increase in body weight correlated with an increase in lean mass and visceral adiposity. Histological examination revealed adipocyte hypertrophy in white epididymal fat and interscapular brown fat in KAEKI vs. WT mice at 17 wpn. Metabolic profiling indicated that KAEKI mice had dyslipidemia and hyperleptinemia but no significant changes in glucose metabolism. Energy balance analyses revealed that hyperphagia preceded weight gain in KAEKI mice.
**Conclusion:** These data suggest that the RGD region of BSP affects energy metabolism by regulating food intake, with further studies warranted to uncover the underlying mechanisms.

KEYWORDS
mineralized tissues, arginine-glycine-aspartic acid (RGD), bone sialoprotein, metabolic activity, obesity, endocrinology, hyperphagia, extracellular matrix protein

## Introduction

Accumulated data suggest that several proteins associated with mineralized tissues, including proteins containing arginine-glycine-aspartic acid (RGD) region, affect energy metabolism (1). For instance, osteocalcin, a bone secretory protein, has been reported to act in an endocrine capacity to regulate energy metabolism within adipocytes, hepatocytes, and pancreatic beta cells (2, 3), and osteopontin, a bone associated RGD

containing secretory glycoprotein, has been reported to affect insulin tolerance (4, 5). As a member of the SIBLING (small integrin-binding ligand, N-linked glycoprotein) family, bone sialoprotein (BSP) contains several highly conserved functional motifs, an N-terminal collagen-binding domain, a poly-glutamic acid (poly-E) sequences that nucleate hydroxyapatite, and a C-terminal RGD-integrin binding domain known to promote cell adhesion, migration, and signaling (6). Data from studies using BSP-deficient mice reveal that BSP is a modulator of mineralization (7–13). In brief, BSP-deficient mice have tooth/bone phenotype with alterations in bone homeostasis and mineralization (hypomineralized) and defects in the region of the periodontium, to include impairments in formation of cementum and surrounding alveolar bone, resulting in a disorganized periodontal ligament (PDL) region, malocclusion and exfoliation of teeth, similar to mice and humans with alkaline phosphatase mutations (14–16). To define the role of the RGD domain of BSP in controlling periodontal tissues, we generated BSP-KAE knock-in (*Ibsp*$^{KAE/KAE}$, hereafter KAEKI) mice by substituting a non-function KAE (lysine-alanine-glutamic acid) sequence for the RGD motif. The results showed an important role of the RGD region of BSP in forming and maintaining the PDL but not in promoting mineralization (6). During our studies with these mice, we noted that the KAEKI mice gained more weight than controls as they aged. This was an unexpected finding as the BSP-deficient mice had lower body weight and size than their wild type (WT) counterparts with no difference in percentage of fat mass between the two genotypes (8). Thus, it appeared that, beyond bones and teeth, the BSP-RGD region may play a role in systemic metabolic activity.

This observation led us to initiate studies to further elucidate the role of the RGD region of BSP in modulating metabolic activity. Importantly, obesity is a major health problem worldwide as it is associated with a number of chronic diseases, including type 2 diabetes, dyslipidemia, cardiovascular diseases and other disorders (17). The fundamental cause of obesity is a long-term energy imbalance between caloric intake and energy expenditure. Alterations of glucose and lipid metabolism have also been reported to influence bone homeostasis (18). Skeletal tissue growth and remodeling are energy consuming processes tightly coupled with the regulation of systemic energy metabolism and reproduction (19). Numerous hormones, such as estrogen, testosterone, parathyroid hormone, insulin, adipokines (e.g., leptin, resistin, adiponectin, tumor necrosis factor-α), vitamin D, as well as neuropeptides modulate bone metabolic activity (18, 20–26). Further, bone marrow adipose tissue, located in close proximity to skeletal lineage cells, has been shown to affect bone metabolism (27–30). Specifically, expansion of this depot, observed with aging, obesity, diabetes, and anorexia nervosa, is often inversely associated with bone mineral density. Bone marrow adipocytes and osteoblasts share a common precursor, mesenchymal stem cells, and thus an imbalance between adipogenesis and osteogenesis, such as a consequence of pathological conditions, may contribute to bone loss.

Added to the growing evidence that specific factors control cell fate toward an adipocyte vs. osteoblast pathway, there exists credible evidence that proteins produced by mineralized tissues, including several RGD containing proteins, affect the activity of tissues at distant sites (1, 20, 31–34). However, the specific role of these proteins at distant sites is not fully understood. Therefore, in this study we utilized a KAEKI mouse model to examine the role of the BSP-RGD region in systemic metabolic activity.

# Materials and methods

## Mice

Animal studies were approved by the NIAMS and NIDDK Animal Care and Use Committees (NIH, Bethesda, MD). KAEKI mice (previously reported as *Ibsp*$^{KAE/KAE}$ mice) were generated by CRISPR/Cas9 as reported previously (6). WT and KAEKI mice were maintained on a C57BL/6 background as described previously (6). Mice were housed at ∼22°C with a 12–12 h light-dark cycle and fed soft gel (DietGel® 31M, 1.91 kcal/g, ClearH$_2$O, Inc, Westbrook, ME) and normal chow (NIH-07, 3.1 kcal/g, Envigo Inc, Madison, WI) diet to ensure that malocclusion, attributable to the impaired periodontal complex reported in global BSP knockout mice, did not occur and thereby affect food intake (35). For consistency, male mice were used for all experiments. A cross-sectional study was performed to measure body weight from 1 to 17 weeks postnatal (wpn), using group housed mice, which were randomly selected throughout the 17 weeks ($n = 4$ per genotype for each time point). A separate cohort of group-housed mice was used for measuring body weight, body length, fat pad and liver weights at 17 wpn, as well as for histological analysis of epididymal and brown fat. Another cohort of singly housed mice was used for measuring energy balance, insulin tolerance and glucose tolerance.

## Measurement of body composition, body length, food intake and energy expenditure

WT ($n = 5$) and KAEKI ($n = 6$) mice were singly housed from 6 till 15 weeks postnatal (wpn). Body weight, body composition, and caloric intake were measured once a week. Total metabolizable caloric intake was calculated from the combined intake of chow and soft gel diets. Body composition (fat mass and fat-free mass) was measured by time domain EchoMRI 3-in-1 (Echo Medical Systems, Houston, TX). Energy expenditure was estimated from the metabolizable caloric intake, corrected for the change in caloric content of the mouse (from the change in body composition over the measurement interval using caloric equivalents of fat mass 9.4 kcal/g and fat-free mass 1.0 kcal/g) (36). Body length (nose-to-anus distance, mm) was assessed immediately after euthanasia at 17 wpn. Body mass index (BMI) was calculated as body weight (kg)/body length$^2$ (m$^2$).

## Insulin and glucose tolerance tests

Insulin Tolerance Test (ITT) was performed at 16 wpn by injecting nonfasted mice with insulin (HumulinR, EliLily,

Indianapolis, IN, 0.75 u/kg, i.p.). Tail blood glucose concentrations were measured at 0, 15, 30, 45, 60 min using glucose meter Contour (Ascensia, Parsippany, NJ). Glucose Tolerance Test (GTT) was conducted at 17 wpn by injecting mice with 20% glucose (2 g/kg, i.p.), following an overnight (16 h) fast, with blood glucose measured at 0, 15, 30, 60, 120 min. ITT and GTT tests were performed on the cohort of mice used for body composition and energy balance analyses. HOMA-IR (Homeostasis Model Assessment of Insulin Resistance) index was calculated as described previously (37).

## Tissue/blood collection and analyses

Another cohort of mice was prepared for blood and tissue analyses. Nonfasted mice were euthanized at 17 wpn by cervical dislocation, followed by collection of blood directly from the heart. Epididymal fat pad (white fat; WT $n = 6$, KAEKI $n = 6$), interscapular brown fat (WT $n = 5$, KAEKI $n = 5$), and liver (WT $n = 6$, KAEKI $n = 6$) were harvested after blood collection. Percent (%) epididymal fat pad and % liver were calculated by dividing tissue weight by body weight per mouse (WT $n = 6$, KAEKI $n = 6$).

Plasma chemistry tests: glucose (WT $n = 11$ KAEKI $n = 12$), triglycerides (WT $n = 10$, KAEKI $n = 12$), and cholesterol (WT $n = 10$, KAEKI $n = 12$) were performed by the Department of Laboratory Medicine, NIH Clinical Center. Insulin (WT $n = 8$, KAEKI $n = 10$) and leptin (WT $n = 7$, KAEKI $n = 8$) levels in sera were measured by Enzyme-Linked Immunosorbent Assay according to the manufacture's protocol (Mouse Leptin ELISA Kit, RAB0334, Sigma-Aldrich; Ultra-sensitive mouse insulin ELISA, #90080, Crystal Chem).

## Histology

Tissues were fixed in 10% neutral buffered formalin for 24 h and paraffin embedded for serial 5$\mu$m sections. Hematoxylin and eosin (H&E) staining was conducted with Harris' Hematoxylin and Eosin Y 1% Alcoholic solution (Thermo Fisher Scientific, Waltham, MA) as described previously (38). For measurement of white adipocyte size, 20 × magnification H&E staining images of epididymal fat pat were measured manually using Rebel Hybrid Microscope (ECHO, San Diego, CA) and cell size was expressed in $mm^2$ ($n = 4$). For measurement of intrascapular brown adipocyte size, 60 × magnification H&E staining images were analyzed using ImageJ (NIH) and data were expressed as percent of area filled with lipid ($n = 5$).

## Statistical analysis

Results are expressed as mean ± standard deviation. Data were analyzed using *t*-test (Prism v.7.04, GraphPad Software, La Jolla, CA). For all tests, $\alpha = 0.05$.

# Results

## Mice lacking BSP-RGD region exhibit obesity with age

To examine the role of the BSP-RGD in systemic metabolic activity we first followed body weight changes in KAEKI and WT mice during development (**Figure 1A**). Growth rates were similar in both genotypes up to 13 wpn. However, beyond that point, KAEKI mice gained weight more rapidly and by 17 wpn were approximately 17% heavier than controls with no difference in body lengths between genotypes (**Figures 1B, D**). Consistent with significantly increased body weight, KAEKI mice developed more visceral fat (**Figure 1C**), had higher BMI (**Figure 1F**), and displayed significantly larger (56%) epididymal fat pats (**Figures 1G–I**) than WT mice. Liver weight was not significantly different between genotypes (**Figures 1J–L**). Taken together, these data indicate that KAEKI mice develop mild obesity with age. We also noted a difference in body weight of WT mice depending on the cohort used (see **Figure 1A** vs. **Figure 1D**), however, this did not alter the significant difference in body weight between WT vs. KAEKI mice.

## KAEKI mice display adipocyte hypertrophy

Expansion of adipose tissue occurs through an increase in adipocyte cell size (hypertrophy) and/or cell number (hyperplasia), with hypertrophic expansion of white fat associated with more severe metabolic dysfunction (39). Therefore, we next examined the histological appearance of white fat (energy storage tissue) and brown fat (thermogenic tissue). The size of both white and brown adipocytes was increased in KAEKI mice compared to WT mice (**Figures 2A, B**). There were also notable differences in the appearance of brown adipocytes. Typically, wild-type brown adipocytes contained multiple small lipid droplets. In contrast, the KAEKI brown fat adipocytes appeared heterogeneous in size, containing predominantly large fat droplets, a phenotype often observed in obese mice with reduced cold-induced thermogenesis (40) (**Figure 2B**).

## KAEKI mice display dyslipidemia and hyperleptinemia

The observed obesity, along with hypertrophy of white adipocytes in KAEKI mice, led us to hypothesis that lack of the BSP-RGD region affects systemic metabolism. Sera analyses revealed that circulating levels of triglycerides and cholesterol were significantly increased in KAEKI mice, indicating a dysregulation of lipid metabolism (**Figures 3A, B**). Levels of leptin, an adipokine produced in proportion to fat mass and a regulator of energy balance by inhibiting food intake, were also significantly increased (**Figure 3C**). However, nonfasted serum glucose and insulin levels, HOMA-IR (insulin resistance index),

10.3389/fdmed.2023.1124084

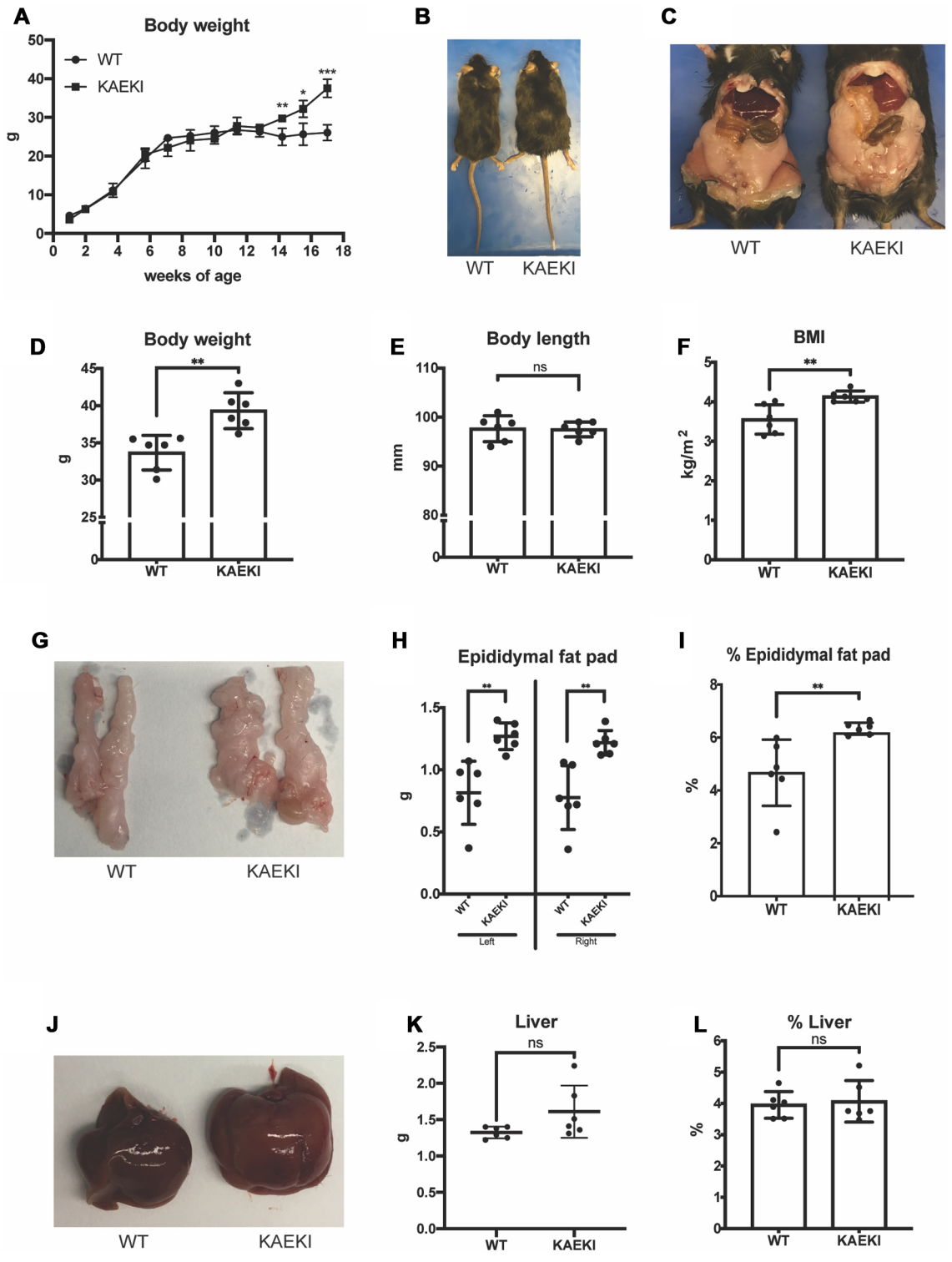



FIGURE 1
Mice lacking the BSP-RGD region develop obesity as they grow. (**A**) Body weights of WT ($n = 4$) and KAEKI ($n = 4$) mice from 1 to 17 wpn. Data were collected from a cross-sectioned study ($n = 4$/genotype/time point). (**B**) Dorsal view of representative WT and KAEKI mice at 17 wpn. (**C**) Ventral view of representative WT and KAEKI mice at 17 wpn after removal of abdominal walls. (**D–E**). Body weight and body length measured in a different set of mice ($n = 6$/group). (**F**) BMI at 17wpn ($n = 6$/group). (**G–I**) Appearance, weight, and % weight of epidydimal fat pads at 17 wpn ($n = 6$/group). (**J–L**) Appearance, weight, and % weight of liver at 17 wpn ($n = 6$/group). Results are expressed as mean ± standard deviation. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns = not significant by *t*-test. Male mice were used for all experiments.

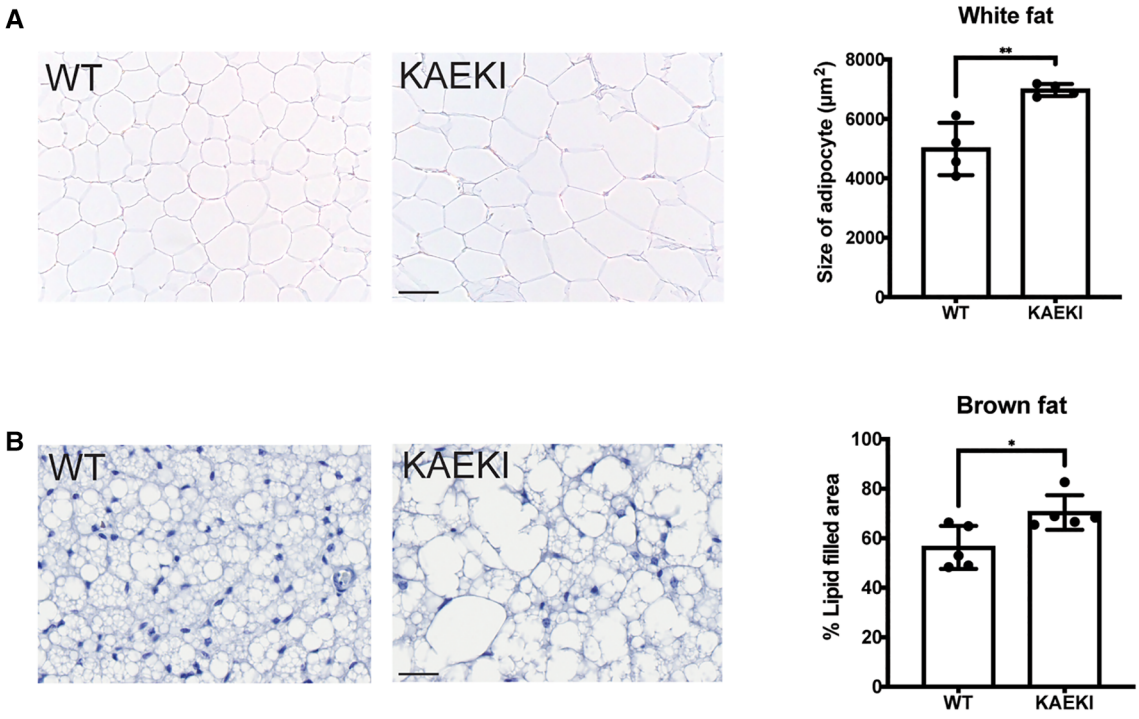



FIGURE 2
KAEKI mice display adipocyte hypertrophy. Histological appearance (H&E staining) of white (epidydimal) fat (A) and interscapular brown fat (B) in WT and KAEKI mice at 17 wpn. For brown fat, relative cell size is expressed as % area filled with lipid. Results are expressed as mean ± standard deviation. *$P < 0.05$; **$P < 0.01$ by *t*-test. Scale bar:100 μm for white fat and 20 μm for brown fat. Male mice were used for all experiments.

insulin tolerance and glucose tolerance were not significantly different between genotypes (Figures 3D–H). Thus, mice lacking BSP-RGD signaling display dyslipidemia and hyperleptinemia with no significant changes in systemic glucose metabolism.

## KAEKI mice display hyperphagia

Obesity results from an imbalance between energy intake and energy expenditure. To gain further insight into the cause of obesity in KAEKI mice, we analyzed energy balance in a separate cohort of mice by monitoring caloric intake and changes in body composition over a period of 9 weeks. Consistent with previous findings, KAEKI mice showed significantly increased body weight (Figure 4A); however, the noted increase in fat mass did not reach significance due to a large variation in individual mice ($p = 0.15$, Figures 4B, C). KAEKI mice also had significantly higher lean mass volume vs. WT mice, although % lean mass, a proportion of lean mass to total body weight, in KAEKI mice tended to be lower than controls (Figures 4C, D), suggesting that both fat and lean mass contribute to the increased weight gain in mice lacking BSP-RGD signaling.

To calculate total caloric intake, we measured cumulative consumption of soft gel diet and chow diet. Both KAEKI and WT mice obtained most of their calories from the soft diet (81% and 86%, respectively, at 15 wpn) with no significant difference between genotypes (Figure 5A). In contrast, the intake of chow was 58% higher in KAEKI mice compared to controls by 15 wpn (Figure 5B), leading to 16% higher total caloric intake by 15 wpn (Figure 5C). This difference was noted early on, 7 wpn, where KAEKI mice had a 12% higher total caloric intake vs. controls. Further, estimated total energy expenditure was slightly higher in the KAEKI mice than WT mice, consistent with their increased lean mass (Figure 5D). Taken together, these data suggest that hyperphagia, but not hypometabolism, was the primary cause of obesity in KAEKI mice.

# Discussion

An intriguing finding of accelerated weight gain in aging KAEKI mice spurred us to examine the role of the BSP-RGD region in metabolic activity. When we first made this observation, we noted increased body weight in both male and female mice, however decided to focus on male mice at this time. Here we show that male mice lacking BSP-RGD signaling develop mild, adult-onset obesity associated with hyperphagia, increased lean mass and visceral adiposity, and adipocyte hypertrophy. Although obesity is commonly associated with metabolic syndrome, including hyperglycemia, insulin resistance and abnormal circulating cholesterol or triglyceride levels, KAEKI mice displayed only a small elevation of serum cholesterol and triglyceride levels and no significant changes in glucose metabolism, consistent with the relatively mild obesity observed in KAEKI mice.

The body weight phenotype of KAEKI mice is strikingly different from the phenotype of BSP null mice, which exhibit

10.3389/fdmed.2023.1124084

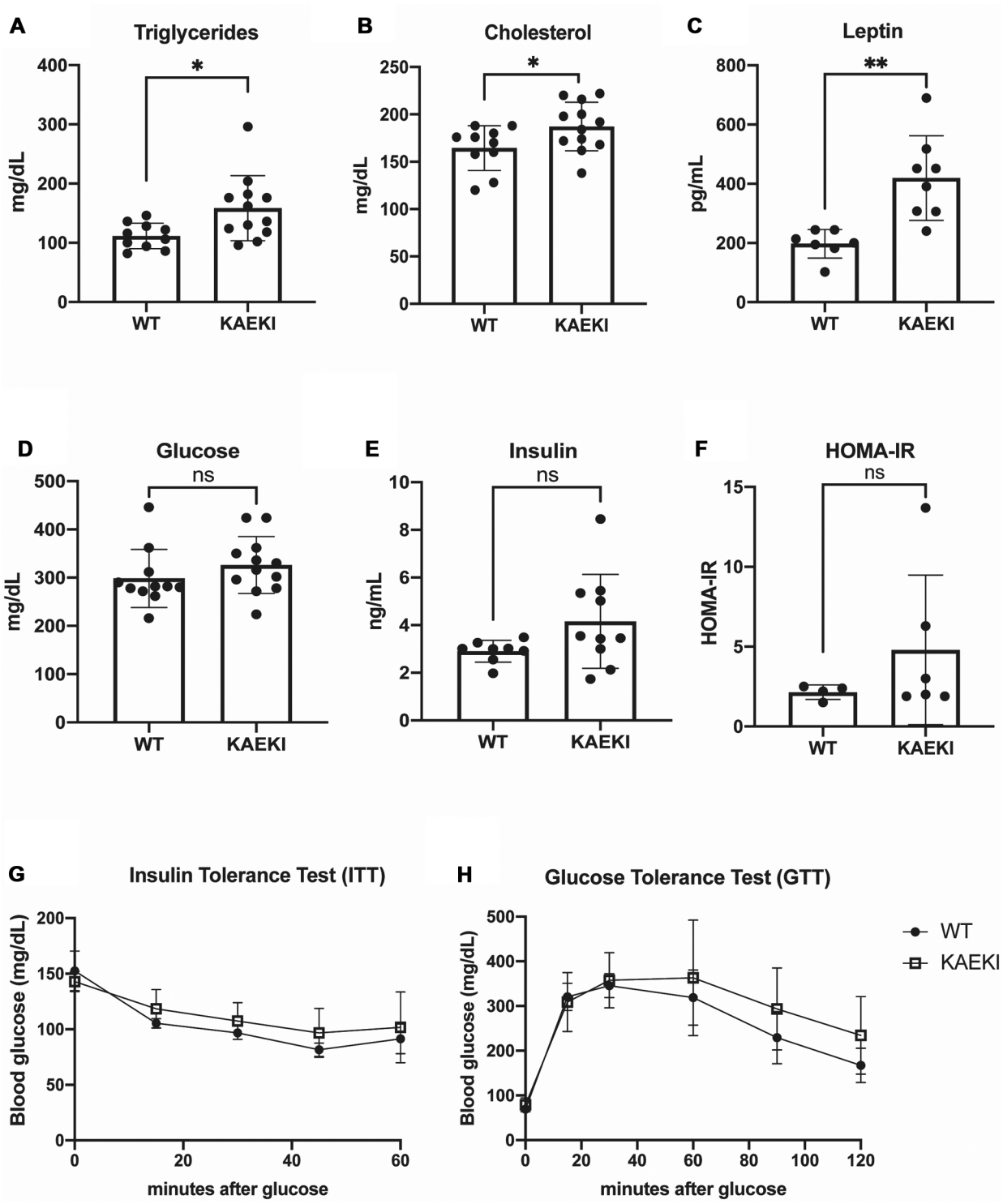



FIGURE 3
KAEKI mice display dyslipidemia and hyperlipidemia. (**A–E**) Blood samples were obtained by cardiac puncture at 17 wpn from WT and KAEKI mice. (**A**) Total triglyceride: WT ($n = 10$) and KAEKI ($n = 12$). (**B**) Total cholesterol: WT ($n = 10$) and KAEKI ($n = 12$). (**C**) Leptin:WT ($n = 7$) and KAEKI ($n = 8$). **D**. Glucose:WT ($n = 11$) and KAEKI ($n = 12$). **E**. Insulin:WT ($n = 8$) and KAEKI ($n = 10$). **F**. HOMA-IR performed at 17 wpn: WT ($n = 4$) and KAEKI ($n = 6$). (**G**) Insulin tolerance test (ITT) performed at 16 wpn: WT ($n = 5$) and KAEKI ($n = 6$). **H**. Glucose tolerance test (GTT) performed at 17 wpn: WT ($n = 5$) and KAEKI ($n = 6$). Results are expressed as mean ± standard deviation. $^*P < 0.05$; $^{**}P < 0.01$; ns = not significant by *t*-test. Male mice were used for all experiments.

lower body weight and size than their WT littermates under standard chow conditions, with no difference in percentage of fat mass between the genotype (8). Feeding BSP null mice a soft diet improved malocclusion, attributed to a severe periodontal phenotype, and normalized their body weight and long bone length, suggesting that malocclusion might be the primary reason

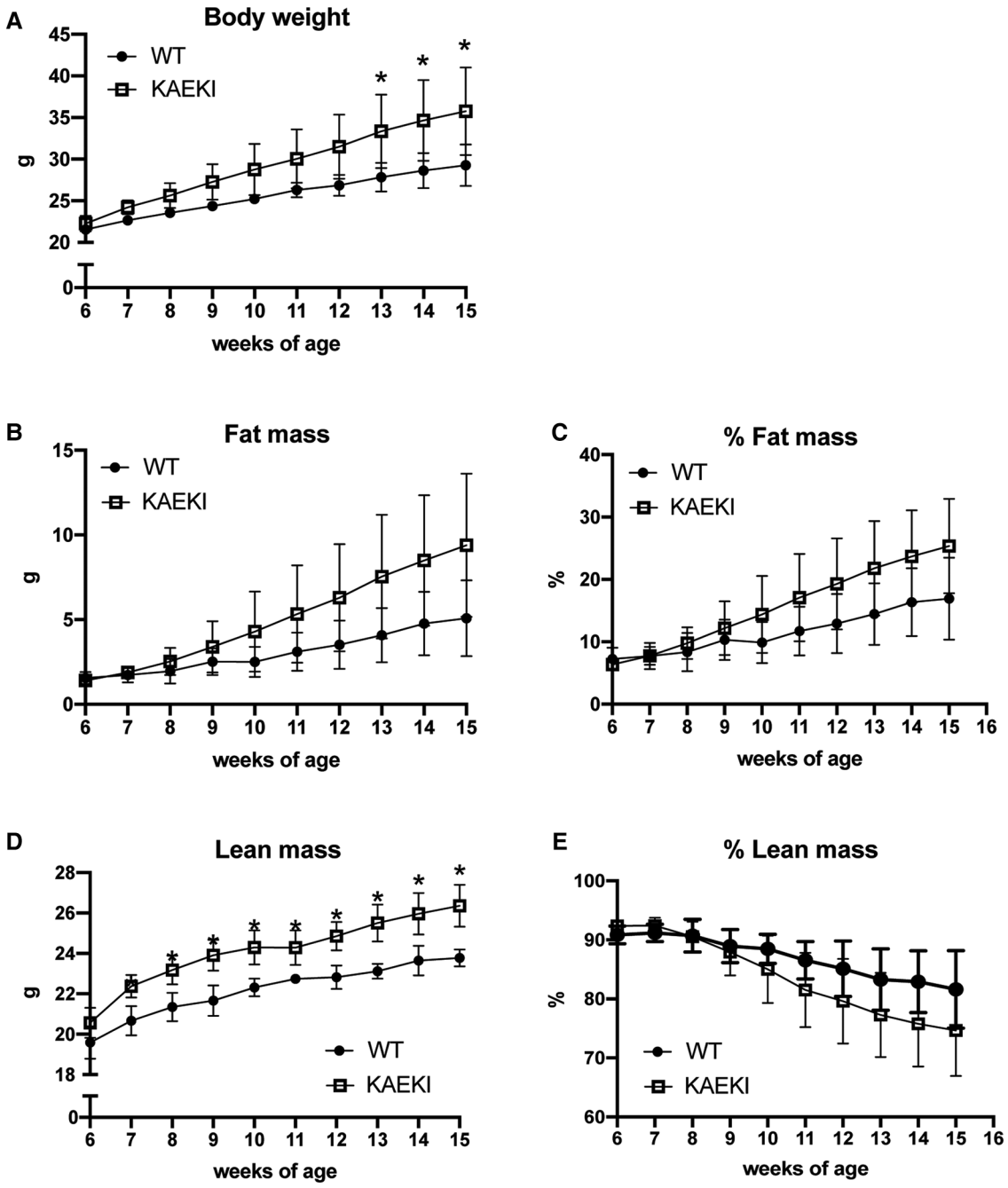



FIGURE 4
Lack of the BSP-RGD region alters body composition. Body weight (A), fat mass (B), % fat mass (C), lean mass (D), and % lean mass (E) were measured weekly in a cohort of WT ($n = 5$) and KAEKI ($n = 6$) mice. Results are expressed as mean ± standard deviation. *$P < 0.05$ by $t$-test. Male mice were used for all experiments.

for the reduced weight gain in the BSP deficient mice maintained on a hard diet (35). Therefore, since we did not have any prior knowledge of the role of the BSP-RGD in maintaining normal occlusion, all mice in this study were given *ad libitum* access to a soft diet and a regular rodent chow, although we recognize that the texture of the diet may affect preference for chow vs. soft gel diet. As we reported previously (6), compared to the BSP null mice (10), the KAEKI mice had a mild periodontal phenotype including a disorganized and dysfunctional PDL and increased osteoclasts along the alveolar bone surface, without disruption of the tooth and bone formation. Even the mild PDL phenotype exhibited in KAEKI mice may affect the response to occlusal loads during mastication (41), however we do not believe this is the case since KAEKI mice consumed equal amounts of the soft diet and more chow diet vs. WT mice without developing malocclusion. For future studies,

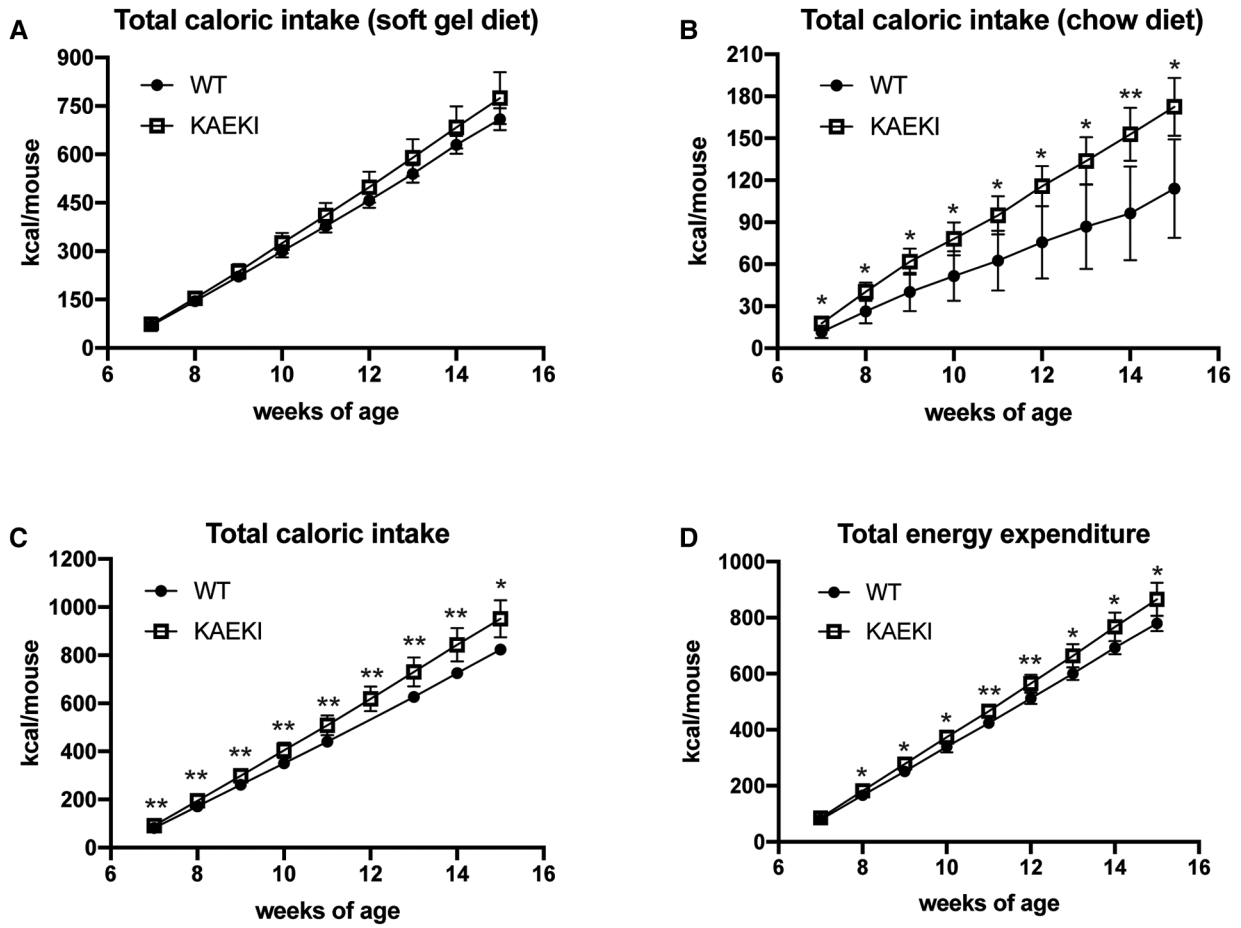



FIGURE 5
KAEKI mice exhibit hyperphagia. Intake of soft gel (A) and chow (B) diets was measured weekly in the cohort of WT ($n = 5$) and KAEKI ($n = 6$) mice used for body composition analysis (see Figure 4). Data are expressed as cumulative caloric intake. (C) Total caloric intake is a combined intake of soft gel and chow diets. (D) Total energy expenditure estimated from total caloric intake, corrected for the change in caloric content of the mouse. Results are expressed as mean ± standard deviation. *$P < 0.05$; **$P < 0.01$; ns = not significant by $t$-test. Male mice were used for all experiments.

standard chow diet will be considered as the diet for both genotypes.

Obesity results from a long-term imbalance between energy intake and energy expenditure, which may be caused by a combination of overconsumption of highly caloric and palatable foods, low physical activity and reduced basal metabolism (17). Studies in mice and humans showed that obesity and aging are also associated with reductions in amount and activity of thermogenic adipose tissue (42). The histological appearance of brown fat in KAEKI mice, including adipocyte hypertrophy, is consistent with the appearance of dormant brown fat observed in obese mice deficient in cold-induced thermogenesis (40). In mice housed at room temperature (22°C), cold induced thermogenesis (mainly mediated by brown fat) accounts for about 30% of total energy expenditure (40). Thus, impaired brown fat thermogenesis can potentially reduce total energy expenditure and lead to weight gain in a mouse. However, the estimated total energy expenditure was slightly higher, not lower, in KAEKI mice than in WT mice, consistent with their increased lean mass. In contrast, KAEKI mice showed significantly increased caloric intake as early as 7 wpn, weeks before detectable changes in body weight gain. Interestingly, the increase of total caloric intake observed in KAEKI mice was mainly driven by higher consumption of a chow diet, which has ∼60% higher caloric density than a soft gel diet. In designing future studies, it would be of value to explore how KAEKI mice respond to high fat or high sugar diets. Taken together, our data suggest that hyperphagia, but not hypometabolism, is the primary cause of obesity in mice lacking BSP-RGD signaling.

One outlier in concluding a KAEKI hyperphagia phenotype is our sera analyses, which revealed that KAEKI mice had significantly increased leptin levels. Leptin, a hormone produced by adipocytes in proportion to fat mass, regulates energy balance primarily by inhibiting food intake (43, 44). Elevated serum leptin in the presence of obesity could be a marker of reduced leptin sensitivity (45) and may contribute to the obesity phenotype of KAEKI mice. However, since these mice displayed hyperphagia prior to detectable changes in fat mass, it is unlikely that hyperleptinemia is a primary cause of obesity in this model.

Skeletal tissue growth and remodeling are energy consuming processes tightly coupled with regulation of energy metabolism and reproduction (19). Osteopontin, another bone secreted RGD containing protein, is considered to be associated with obesity, insulin resistance and type 2 diabetes with a reported vital role in modulating inflammation within many tissues including adipose tissue (4, 5, 46). Moreover, osteocalcin, a protein specifically expressed by osteoblasts, has been reported to regulate energy metabolism *via* effects on adipocytes, hepatocytes, and pancreatic beta cells (2, 3). While further studies are needed to better understand the influence of the RGD region of BSP on metabolic activity, the results reported here add to existing evidence that proteins within mineralized tissues influence metabolic activity at other sites (1). The metabolic homeostasis of higher organisms relies on precise sensing of the energy state of the body and a coordinated response of multiple organs to nutritional demands and environmental changes. The central nervous system plays an important role in regulating all aspects of metabolism, including energy intake, utilization, and storage (47–49). One-way peripheral tissues communicate with the brain is *via* secreted factors, including proteins, hormones, cytokines, and metabolites (26, 39, 50–53). BSP is a multifunctional extracellular matrix protein abundant in bone, cementum, and dentin (54–56). Although low levels of *Ibsp* mRNA have been detected in the mouse brain (https://www.ncbi.nlm.nih.gov/gene/3381, http://www.informatics.jax.org, https://www.ebi.ac.uk), there is no evidence of BSP expression in the areas of the brain regulating energy metabolism. A small amount of BSP is found in the circulation (57) but it is likely to be just a marker of bone turnover as BSP is not known to have an endocrine function. Thus, the metabolic phenotype of KAEKI mice is more likely caused by impaired BSP-RGD signaling within mineralized tissues rather than its direct effect at distal sites. For example, by affecting the levels of proteins within mineralized tissues, reported to affect energy metabolism (1, 20, 31, 32).

In this regard, as mentioned above, existing data provide credible evidence that proteins produced by mineralized tissues, including several RGD containing proteins, affect the metabolic activity of tissues at distant sites, including regulation of body weight, energy expenditure, insulin secretion and insulin sensitivity (1, 20, 31–33). To date, the osteoblast-derived lipocalin-2 is the only known bone-derived factor that has been shown to regulate food intake directly by activating melanocortin 4 receptor-dependent anorexigenic pathway in the hypothalamus (58). Further studies are needed to determine if expression of lipocalin 2 and other known bone-derived factors is altered in mice lacking BSP-RGD signaling.

Taken together, our data suggest that, beyond its role in bones and teeth, the RGD region of BSP contributes to systemic metabolic activity by controlling food intake, consequently increasing caloric storage within adipocytes, resulting in white and brown fat hypertrophy and overall gain of fat and lean mass. Further studies are warranted to determine the mechanisms by which the RGD region of BSP, as well as other RGD containing proteins, modulate systemic metabolic activity.

## Data availability statement

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

## Ethics statement

The animal study was reviewed and approved by National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) Animal Care and Use Committees (NIH, Bethesda, MD) and National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Animal Care and Use Committees (NIH, Bethesda, MD).

## Author contributions

KN contributed to experimental designing, data curation and analysis, and writing and finalizing the manuscript. AN, JMT, BDK, and YM contributed to data curation and analysis. MJS, and OG contributed equally to designing, writing, and finalizing the manuscript. All authors agree to be accountable for the content of the work. All authors contributed to the article and approved the submitted version.

## Funding

KN, AN, and MJS were supported by NIAMS/NIH intramural program. KN was supported by JST, the establishment of university fellowships towards the creation of science technology innovation, Grant Number JPMJFS2102. AN was supported by the Japan Society for the Promotion of Science (JSPS Research Fellowship for Japanese Biomedical and Behavioral Researchers at the National Institutes of Health). OG was supported by NIDDK/NIH intramural program.

## Acknowledgments

We thank NIH and JST, and JSPS for the support of this project.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated

organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

## References


1. Nagasaki K, Gavrilova O, Hajishengallis G, Somerman MJ. Does the RGD region of certain proteins affect metabolic activity? *Front Dent Med*. (2022) 3:974862. doi: 10.3389/fdmed.2022.97486

2. Wei J, Karsenty G. An overview of the metabolic functions of osteocalcin. *Curr Osteoporos Rep*. (2015) 13(3):180–5. doi: 10.1007/s11914-015-0267-y

3. Otani T, Mizokami A, Kawakubo-Yasukochi T, Takeuchi H, Inai T, Hirata M. The roles of osteocalcin in lipid metabolism in adipose tissue and liver. *Adv Biol Regul*. (2020) 78:100752. doi: 10.1016/j.jbior.2020.100752

4. Kiefer FW, Zeyda M, Gollinger K, Pfau B, Neuhofer A, Weichhart T, et al. Neutralization of osteopontin inhibits obesity-induced inflammation and insulin resistance. *Diabetes*. (2010) 59(4):935–46. doi: 10.2337/db09-0404

5. Kahles F, Findeisen HM, Bruemmer D. Osteopontin: a novel regulator at the cross roads of inflammation, obesity and diabetes. *Mol Metab*. (2014) 3(4):384–93. doi: 10.1016/j.molmet.2014.03.004

6. Nagasaki K, Chavez MB, Nagasaki A, Taylor JM, Tan MH, Ma M, et al. The bone sialoprotein RGD domain modulates and maintains periodontal development. *J Dent Res*. (2022) 101:220345221100794. doi: 10.1177/00220345221100794

7. Bouleftour W, Boudiffa M, Wade-Gueye NM, Bouët G, Cardelli M, Laroche N, et al. Skeletal development of mice lacking bone sialoprotein (BSP)–impairment of long bone growth and progressive establishment of high trabecular bone mass. *PLoS One*. (2014) 9(5):e95144. doi: 10.1371/journal.pone.0095144

8. Malaval L, Wade-Guéye NM, Boudiffa M, Fei J, Zirngibl R, Chen F, et al. Bone sialoprotein plays a functional role in bone formation and osteoclastogenesis. *J Exp Med*. (2008) 205(5):1145–53. doi: 10.1084/jem.20071294

9. Foster BL, Soenjaya Y, Nociti FH, Holm E, Zerfas PM, Wimer HF, et al. Deficiency in acellular cementum and periodontal attachment in bsp null mice. *J Dent Res*. (2013) 92(2):166–72. doi: 10.1177/0022034512469026

10. Foster BL, Ao M, Willoughby C, Soenjaya Y, Holm E, Lukashova L, et al. Mineralization defects in cementum and craniofacial bone from loss of bone sialoprotein. *Bone*. (2015) 78:150–64. doi: 10.1016/j.bone.2015.05.007

11. Bouleftour W, Bouet G, Granito RN, Thomas M, Linossier MT, Vanden-Bossche A, et al. Blocking the expression of both bone sialoprotein (BSP) and osteopontin (OPN) impairs the anabolic action of PTH in mouse calvaria bone. *J Cell Physiol*. (2015) 230(3):568–77. doi: 10.1002/jcp.24772

12. Bouleftour W, Juignet L, Verdière L, Machuca-Gayet I, Thomas M, Laroche N, et al. Deletion of OPN in BSP knockout mice does not correct bone hypomineralization but results in high bone turnover. *Bone*. (2019) 120:411–22. doi: 10.1016/j.bone.2018.12.001

13. Shaarawy M, Hasan M. Serum bone sialoprotein: a marker of bone resorption in postmenopausal osteoporosis. *Scand J Clin Lab Invest*. (2001) 61(7):513–21. doi: 10.1080/003655101753218274

14. Gasque KC, Foster BL, Kuss P, Yadav MC, Liu J, Kiffer-Moreira T, et al. Improvement of the skeletal and dental hypophosphatasia phenotype in alpl-/- mice by administration of soluble (non-targeted) chimeric alkaline phosphatase. *Bone*. (2015) 72:137–47. doi: 10.1016/j.bone.2014.11.017

15. Kramer K, Chavez MB, Tran AT, Farah F, Tan MH, Kolli TN, et al. Dental defects in the primary dentition associated with hypophosphatasia from biallelic ALPL mutations. *Bone*. (2021) 02(143):115732. doi: 10.1016/j.bone.2020.115732

16. Foster BL, Kuss P, Yadav MC, Kolli TN, Narisawa S, Lukashova L, et al. Conditional alpl ablation phenocopies dental defects of hypophosphatasia. *J Dent Res*. (2017) 96(1):81–91. doi: 10.1177/0022034516663633

17. Blüher M. Obesity: global epidemiology and pathogenesis. *Nat Rev Endocrinol*. (2019) 15(5):288–98. doi: 10.1038/s41574-019-0176-8

18. Suzuki A, Minamide M, Iwaya C, Ogata K, Iwata J. Role of metabolism in bone development and homeostasis. *Int J Mol Sci*. (2020) 21(23):8992. doi: 10.3390/ijms21238992

19. Karsenty G, Khosla S. The crosstalk between bone remodeling and energy metabolism: a translational perspective. *Cell Metab*. (2022) 34:805–17. doi: 10.1016/j.cmet.2022.04.010

20. Fulzele K, Riddle RC, DiGirolamo DJ, Cao X, Wan C, Chen D, et al. Insulin receptor signaling in osteoblasts regulates postnatal bone acquisition and body composition. *Cell*. (2010) 142(2):309–19. doi: 10.1016/j.cell.2010.06.002

21. Wee NK, Kulkarni RN, Horsnell H, Baldock PA. The brain in bone and fuel metabolism. *Bone*. (2016) 82:56–63. doi: 10.1016/j.bone.2015.10.020

22. Parimisetty A, Dorsemans AC, Awada R, Ravanan P, Diotel N, Lefebvre d'Hellencourt C. Secret talk between adipose tissue and central nervous system via secreted factors-an emerging frontier in the neurodegenerative research. *J Neuroinflammation*. (2016) 13(1):67. doi: 10.1186/s12974-016-0530-x

23. Thommesen L, Stunes AK, Monjo M, Grøsvik K, Tamburstuen MV, Kjøbli E, et al. Expression and regulation of resistin in osteoblasts and osteoclasts indicate a role in bone metabolism. *J Cell Biochem*. (2006) 99(3):824–34. doi: 10.1002/jcb.20915

24. Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, et al. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell*. (2000) 100(2):197–207. doi: 10.1016/s0092-8674(00)81558-5

25. Lewis JW, Edwards JR, Naylor AJ, McGettrick HM. Adiponectin signalling in bone homeostasis, with age and in disease. *Bone Res*. (2021) 9(1):1. doi: 10.1038/s41413-020-00122-0

26. Yang Q, Vijayakumar A, Kahn BB. Metabolites as regulators of insulin sensitivity and metabolism. *Nat Rev Mol Cell Biol*. (2018) 19(10):654–72. doi: 10.1038/s41580-018-0044-8

27. Horowitz MC, Berry R, Holtrup B, Sebo Z, Nelson T, Fretz JA, et al. Bone marrow adipocytes. *Adipocyte*. (2017) 6(3):193–204. doi: 10.1080/21623945.2017.1367881

28. Pachón-Peña G, Bredella MA. Bone marrow adipose tissue in metabolic health. *Trends Endocrinol Metab*. (2022) 33(6):401–8. doi: 10.1016/j.tem.2022.03.003

29. Li J, Liu X, Zuo B, Zhang L. The role of bone marrow microenvironment in governing the balance between osteoblastogenesis and adipogenesis. *Aging Dis*. (2016) 7(4):514–25. doi: 10.14336/AD.2015.1206

30. Ning K, Liu S, Yang B, Wang R, Man G, Wang DE, et al. Update on the effects of energy metabolism in bone marrow mesenchymal stem cells differentiation. *Mol Metab*. (2022) 58:101450. doi: 10.1016/j.molmet.2022.101450

31. Wei J, Ferron M, Clarke CJ, Hannun YA, Jiang H, Blaner WS, et al. Bone-specific insulin resistance disrupts whole-body glucose homeostasis via decreased osteocalcin activation. *J Clin Invest*. (2014) 124(4):1–13. doi: 10.1172/JCI72323

32. Confavreux CB, Levine RL, Karsenty G. A paradigm of integrative physiology, the crosstalk between bone and energy metabolisms. *Mol Cell Endocrinol*. (2009) 310(1–2):21–9. doi: 10.1016/j.mce.2009.04.004

33. Goettsch C, Strzelecka-Kiliszek A, Bessueille L, Quillard T, Mechtouff L, Pikula S, et al. TNAP As a therapeutic target for cardiovascular calcification: a discussion of its pleiotropic functions in the body. *Cardiovasc Res*. (2022) 118(1):84–96. doi: 10.1093/cvr/cvaa299

34. Fulzele K, Lai F, Dedic C, Saini V, Uda Y, Shi C, et al. Osteocyte-Secreted wnt signaling inhibitor sclerostin contributes to beige adipogenesis in peripheral fat depots. *J Bone Miner Res*. (2017) 32(2):373–84. doi: 10.1002/jbmr.3001

35. Soenjaya Y, Foster BL, Nociti FH, Ao M, Holdsworth DW, Hunter GK, et al. Mechanical forces exacerbate periodontal defects in bsp-null mice. *J Dent Res*. (2015) 94(9):1276–85. doi: 10.1177/0022034515592581

36. Ravussin Y, Gutman R, LeDuc CA, Leibel RL. Estimating energy expenditure in mice using an energy balance technique. *Int J Obes (Lond)*. (2013) 37(3):399–403. doi: 10.1038/ijo.2012.105

37. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. (1985) 28(7):412–9. doi: 10.1007/BF00280883

38. Foster BL, Ao M, Salmon CR, Chavez MB, Kolli TN, Tran AB, et al. Osteopontin regulates dentin and alveolar bone development and mineralization. *Bone*. (2018) 02(107):196–207. doi: 10.1016/j.bone.2017.12.004

39. Priest C, Tontonoz P. Inter-organ cross-talk in metabolic syndrome. *Nat Metab*. (2019) 1(12):1177–88. doi: 10.1038/s42255-019-0145-5

40. Himms-Hagen J. Brown adipose tissue thermogenesis and obesity. *Prog Lipid Res*. (1989) 28(2):67–115. doi: 10.1016/0163-7827(89)90009-x

41. Naveh GR, Lev-Tov Chattah N, Zaslansky P, Shahar R, Weiner S. Tooth-PDL-bone complex: response to compressive loads encountered during mastication—a review. *Arch Oral Biol*. (2012) 57(12):1575–84. doi: 10.1016/j.archoralbio.2012.07.006

42. Sakers A, De Siqueira MK, Seale P, Villanueva CJ. Adipose-tissue plasticity in health and disease. *Cell*. (2022) 185(3):419–46. doi: 10.1016/j.cell.2021.12.016

43. Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, et al. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science*. (1995) 269(5223):543–6. doi: 10.1126/science.7624777

44. Friedman JM. Leptin and the endocrine control of energy balance. *Nat Metab*. (2019) 1(8):754–64. doi: 10.1038/s42255-019-0095-y

45. Myers MG, Leibel RL, Seeley RJ, Schwartz MW. Obesity and leptin resistance: distinguishing cause from effect. *Trends Endocrinol Metab*. (2010) 21(11):643–51. doi: 10.1016/j.tem.2010.08.002

46. Icer MA, Gezmen-Karadag M. The multiple functions and mechanisms of osteopontin. *Clin Biochem*. (2018) 59:17–24. doi: 10.1016/j.clinbiochem.2018.07.003

47. Kim KS, Seeley RJ, Sandoval DA. Signalling from the periphery to the brain that regulates energy homeostasis. *Nat Rev Neurosci*. (2018) 19(4):185–96. doi: 10.1038/nrn.2018.8

48. Jais A, Brüning JC. Arcuate nucleus-dependent regulation of metabolism-pathways to obesity and diabetes Mellitus. *Endocr Rev*. (2022) 43(2):314–28. doi: 10.1210/endrev/bnab025

49. Alcantara IC, Tapia APM, Aponte Y, Krashes MJ. Acts of appetite: neural circuits governing the appetitive, consummatory, and terminating phases of feeding. *Nat Metab*. (2022) 4(7):836–47. doi: 10.1038/s42255-022-00611-y

50. Funcke JB, Scherer PE. Beyond adiponectin and leptin: adipose tissue-derived mediators of inter-organ communication. *J Lipid Res*. (2019) 60(10):1648–84. doi: 10.1194/jlr.R094060

51. Liu S, Alexander RK, Lee CH. Lipid metabolites as metabolic messengers in inter-organ communication. *Trends Endocrinol Metab*. (2014) 25(7):356–63. doi: 10.1016/j.tem.2014.05.002

52. Crewe C, Scherer PE. Intercellular and interorgan crosstalk through adipocyte extracellular vesicles. *Rev Endocr Metab Disord*. (2022) 23(1):61–9. doi: 10.1007/s11154-020-09625-x

53. Romero A, Eckel J. Organ crosstalk and the modulation of insulin signaling. *Cells*. (2021) 10(8):2082. doi: 10.3390/cells10082082

54. Bouleftour W, Juignet L, Bouet G, Granito RN, Vanden-Bossche A, Laroche N, et al. The role of the SIBLING, bone sialoprotein in skeletal biology—contribution of mouse experimental genetics. *Matrix Biol*. (2016) 52-54:60–77. doi: 10.1016/j.matbio.2015.12.011

55. Ganss B, Kim RH, Sodek J. Bone sialoprotein. *Crit Rev Oral Biol Med*. (1999) 10 (1):79–98. doi: 10.1177/10454411990100010401

56. Fisher LW, Fedarko NS. Six genes expressed in bones and teeth encode the current members of the SIBLING family of proteins. *Connect Tissue Res*. (2003) 44 (Suppl 1):33–40. doi: 10.1080/03008200390152061

57. Seibel MJ, Woitge HW, Pecherstorfer M, Karmatschek M, Horn E, Ludwig H, et al. Serum immunoreactive bone sialoprotein as a new marker of bone turnover in metabolic and malignant bone disease. *J Clin Endocrinol Metab*. (1996) 81 (9):3289–94. doi: 10.1210/jcem.81.9.8784085

58. Mosialou I, Shikhel S, Liu JM, Maurizi A, Luo N, He Z, et al. Corrigendum: mC4R-dependent suppression of appetite by bone-derived lipocalin 2. *Nature*. (2017) 546(7658):440. doi: 10.1038/nature22808

frontiers | Frontiers in Dental Medicine

TYPE Review
PUBLISHED 11 May 2023
DOI 10.3389/fdmed.2023.1181817



OPEN ACCESS

EDITED BY
Martha J. Somerman,
Frontiers in Dental Medicine, United States

REVIEWED BY
Pamela Gehron Robey,
National Institutes of Health (NIH), United States
Dobrawa Napierala,
University of Pittsburgh, United States
Michael Baker Chavez,
The Ohio State University, United States

*CORRESPONDENCE
Renny T. Franceschi
rennyf@umich.edu

RECEIVED 07 March 2023
ACCEPTED 20 April 2023
PUBLISHED 11 May 2023

CITATION
Franceschi RT, Hallett SA and Ge C (2023) Discoidin domain receptors; an ancient family of collagen receptors has major roles in bone development, regeneration and metabolism. Front. Dent. Med 4:1181817. doi: 10.3389/fdmed.2023.1181817



# Discoidin domain receptors; an ancient family of collagen receptors has major roles in bone development, regeneration and metabolism

Renny T. Franceschi*, Shawn A. Hallett and Chunxi Ge

Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry, Ann Arbor, MI, United States

The extracellular matrix (ECM) niche plays a critical role in determining cellular behavior during bone development including the differentiation and lineage allocation of skeletal progenitor cells to chondrocytes, osteoblasts, or marrow adipocytes. As the major ECM component in mineralized tissues, collagen has instructive as well as structural roles during bone development and is required for bone cell differentiation. Cells sense their extracellular environment using specific cell surface receptors. For many years, specific β1 integrins were considered the main collagen receptors in bone, but, more recently, the important role of a second, more primordial collagen receptor family, the discoidin domain receptors, has become apparent. This review will specifically focus on the roles of discoidin domain receptors in mineralized tissue development as well as related functions in abnormal bone formation, regeneration and metabolism.

KEYWORDS

extracellular matrix, collagen receptor, differentiation, stem cell, bone, cartilage

## 1. Introduction to collagen receptors

As the most abundant class of ECM proteins, collagens provide structural support for connective tissues, skin and, most importantly, bones and teeth, and can convey information about the extracellular mechanical environment via their interaction with cells using specific collagen receptors. The importance of collagen to bone development is well established; collagen synthesis is necessary for differentiation of skeletal progenitors to osteoblasts (1–4) and conditions that interfere with collagen synthesis or structure *in vivo* such as vitamin C deficiency or osteogenesis imperfecta severely disrupt bone development (5–8).

Until recently, it was generally assumed that bone cells interacted with the collagenous ECM exclusively through integrins, the best-known ECM receptors. Through their linkage with the cytoskeleton, integrins are major force transducers linking the ECM microenvironment with cellular functions including nuclear transcription (9). The collagen-binding integrins all have a common β1 subunit and four different alpha subunits to produce α1β1, α2β1, α10β1 and α11β1 integrins, which are all detected in bone (10–13). Disruption of integrin-collagen binding in cell culture using blocking antibodies to specific integrin subunits inhibits osteoblast differentiation of skeletal progenitor cells including preosteoblast cell lines and primary bone marrow cell cultures (12, 14–16). Because of their shared β1 subunit, the overall requirement for collagen-binding integrins in bone was

assessed *in vivo* using conditional inactivation of the β1 integrin gene (*Itgb1*). Using this approach, bone phenotypes of varying severity were observed with the strongest effects of *Itgb1* inactivation being associated with expression of Cre recombinase early in the bone lineage and milder phenotypes seen at later stages. For example, *Itgb1* inactivation in embryonic mesenchymal progenitors using *Twist2-Cre* was associated with severe bone phenotypes and perinatal lethality (17). Disruption at later stages using *Osx-Cre* (preosteoblast stage) reduced skeletal growth, mineralization and mechanical properties, effects that became progressively milder with age while disruption of *Itgb1* with *Bglap-Cre* had only minor effects on skeletal development (17, 18). Similarly, *Itgb1* inactivation in cartilage using *Col2a1-Cre* resulted in perinatal lethality in most pups, stunted cartilage growth and disruption of chondrocyte proliferation and polarity (19). Although in some cases loss of *Itgb1* function severely retarded bone development, in no case was bone formation and mineralization completely disrupted. This shows that some degree of bone formation can occur in the absence of collagen-binding integrins and suggests the involvement of other collagen receptors.

Interestingly, the collagen-binding integrins appeared relatively late in evolutionary history, being first seen with the emergence of chordates (20). In contrast, collagen-like proteins are present in all metazoan species (21). The discoidin domain receptors (DDRs) are a more ancient class of cell-surface collagen binding proteins than integrins. Like collagens, they are present in most invertebrate metazoans including *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Hydra vulgaris* and so could function as collagen receptors before the collagen-binding integrins appeared on the scene. Although functions of DDRs in invertebrates have not been extensively examined, in *C. elegans*, specific DDR functions have been described related to axonal guidance which also requires collagen. Since DDRs have likely functioned as collagen receptors over a much longer period of time than integrins, they may have more primordial functions related to collagen signaling [for review, see reference (22)].

As will be discussed, DDRs are very different from integrins in terms of their interaction with collagens, structure, mechanism of action, tissue distribution and activity in specific cell populations. This review will specifically focus on roles of DDRs in mineralized tissues. However, it should be noted that DDRs also have non-skeletal functions in epithelial and connective tissues and have been linked to several diseases including cancer, fibrosis, and kidney disease that will not be discussed here. The reader is referred to several excellent reviews for a comprehensive treatment of these diverse DDR activities (23–26).

## 2. DDR structure and function

Unlike integrins, which lack intrinsic kinase activity, the DDRs are collagen-activated receptor tyrosine kinases (RTKs) that share homology in their kinase domain with growth factor receptors such as the neurotrophin receptor, TrkA (25, 27, 28). DDRs are named for their homology to the *Dictyostelium discoideum* lectin, discoidin. In mammals, there are two DDR proteins, DDR1 and DDR2, which show different preferences for binding to fibrillar and non-fibrillar collagens. Both DDR1 and 2 bind type I, II, III and V fibrillar collagens. In contrast, DDR1 selectively binds basement membrane type IV collagen while DDR2 binds type X collagen (27–29). The overall structural features of DDR1 and 2 are summarized in **Figure 1**. Starting from the N-terminus, both proteins have an extracellular DS domain, the region of homology with discoidin, a DS-like domain, a juxtamembrane domain, a single pass transmembrane domain, an intracellular juxtamembrane domain and an intracellular kinase domain. DS and DS-like domains and the kinase domain are highly conserved between DDR1 and DDR2. The DS domain distinguishes the DDRs from other RTKs and contains the binding site for triple-helical collagens (31, 32). DDR1 exists in 5 different spliced forms while only a single DDR2 protein has been described. In DDR1, the extracellular and transmembrane domains are shared between all 5 isoforms while there are several differences in the cytoplasmic domains. Two of the 5 DDR1 splice variants lack a functional kinase domain and could potentially act as decoy receptors for the kinase-containing isoforms (25).

Like the collagen-binding integrins, the DDRs only bind to native triple-helical collagens [i.e., thermally denatured collagen cannot serve as a binding substrate (21, 28, 31)]. DDR1 and 2 both bind a 6 amino acid sequence present in fibrillar collagens I-III, GVMGFO, where O is hydroxyproline (33, 34). This same sequence is also recognized by two other collagen-binding proteins, Secreted Protein Acidic and Rich in Cysteine (SPARC) and von Willebrand Factor that have functions in collagen mineralization and the blood coagulation cascade, respectively (35, 36). The GVMGFO sequence is distinct from the motif recognized by collagen-binding integrins which has the consensus sequence, GxOGEx (e.g., GFOGER or GAOGER in fibrillar collagens) (37, 38). Interestingly, in the COL1A1, COL2A1 and COL3A1 chains of types I–III collagen, the O of GVMGFO and the G of GFOGER/GAOGER are separated by 96 amino acid residues, a finding with possible implications concerning coupling between DDRs and integrins (see **Section 6**). The interaction between the DDR2 DS domain and a triple-helical peptide containing the GVMGFO sequence has been examined at atomic resolution using x-ray crystallography (39). These studies identified an amphiphilic binding pocket for the GVMGFO sequence that is conserved between DDR2 and DDR1. One side of this pocket contains apolar amino acid residues (Trp52, Thr56, Asn175, Cys73-Cys177) while the other side contains polar residues forming a salt bridge (Arg105-Glu113, Asp69) (39).

Like other RTKs, the DDRs are ligand-activated tyrosine kinases. However, instead of responding to soluble molecules such as growth factors, the DDRs have high molecular weight triple-helical collagen as a ligand. They differ from classic RTKs in other ways as well. Instead of existing as monomers that dimerize with ligand binding, DDRs are homodimers in the unactivated state (40, 41). Also, instead of being activated by their ligands and undergoing autophosphorylation within seconds to minutes like other RTKs, DDR phosphorylation takes hours and can often persist for days after binding collagen (27, 28). No truly satisfactory explanation for this phenomenon

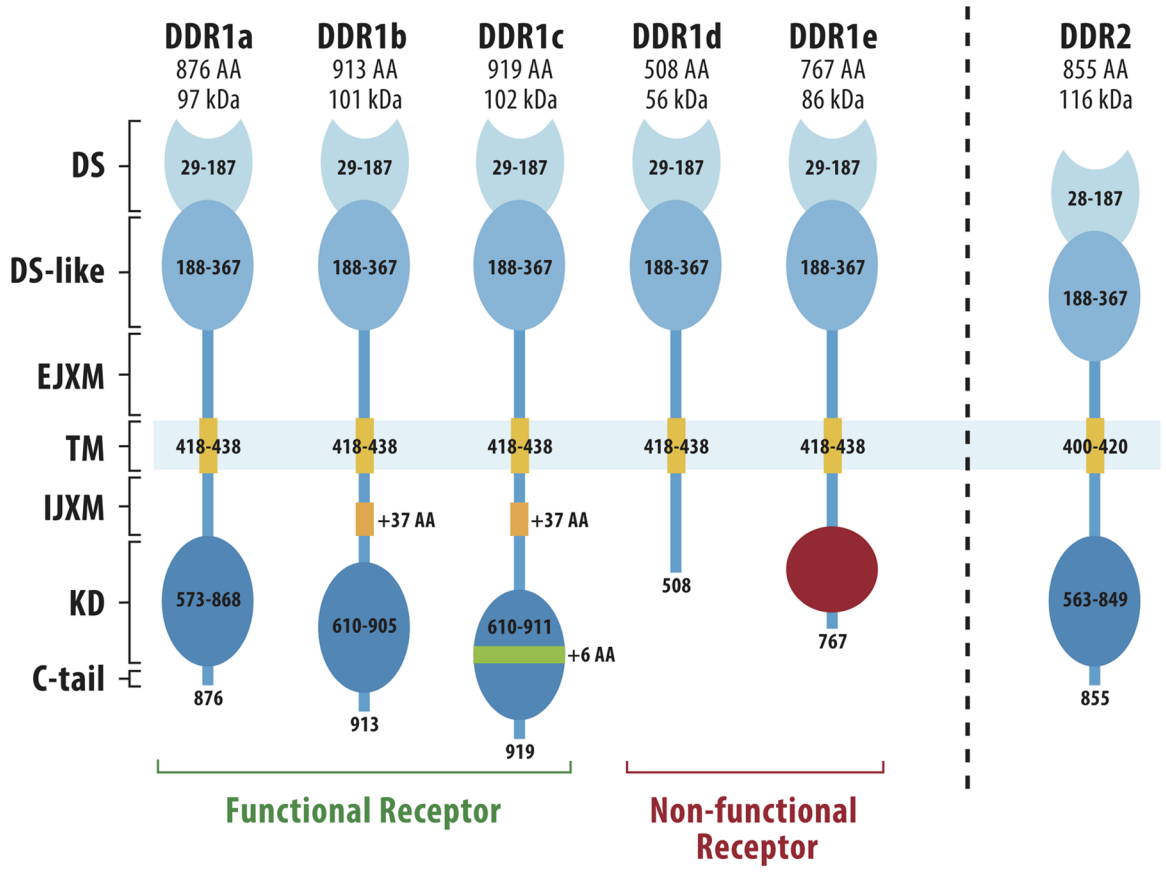



FIGURE 1
Structures of DDR1 and DDR2. DDR1 has 5 different spliced variants (DDR1a-e) while DDR2 exists only as a single protein. N-terminal DS (discoidin) and DS-like (discoidin-like) globular domains are shared by all DDR1 spliced variants and share high homology with the same domains in DDR2. Other regions are an extracellular juxtamembrane domain (EJXM), a transmembrane domain (TM), an intracellular juxtamembrane domain (IJXM), a kinase domain (KD) and a short C-terminal tail. The collagen-binding pocket is contained within the DS domain. Adapted from Rammal et al. (30).

has been advanced although the involvement of secondary cellular processes such as oligomerization or internalization may be important (40, 42). Since DDRs are activated with similar kinetics by small triple-helical peptides containing the GVMGFO core binding sequence, higher order fibrillar structure of native collagen is not required for this unusual behavior (33, 34).

Once activated, DDRs stimulate several downstream signals including ERK1/2 and p38 mitogen-activated protein kinase, phosphatidylinositol-3-kinase/AKT and NF-Kβ pathways. DDRs may also have functions separate from their kinase activities, possibly related to the control of collagen fibrillogenesis and/or orientation (43, 44). It is not the purpose of this review to provide a comprehensive discussion of DDR2 signaling mechanisms as these have been thoroughly reviewed by others [see ref (23, 25)].

## 3. Tissue distribution of DDR1 and DDR2 in mineralized tissues

Initial evaluation of *Ddr1* and *Ddr2* mRNA distribution suggested that *Ddr1* is predominantly expressed in epithelial tissues, smooth muscle and immune cells while *Ddr2* is in connective tissues (45). More recently, tissue distribution was assessed by immunohistochemistry and *in situ* hybridization as well as by using a LacZ knock-in *Ddr2* mutant where a bacterial β-galactosidase gene was inserted into the *Ddr2* locus. The following discussion will emphasize DDR distribution in mineralized tissues.

### 3.1. DDR1

Although an early study that measured DDR1 binding sites in mice using DDR1 extracellular domain fused with alkaline phosphatase showed binding to all skeletal structures, skin and the urogenital tract because of their high collagen content (46), studies that actually measured the tissue distribution of DDR1 protein or mRNA are quite limited. In neonatal and adult mice, DDR1 was localized by immunohistochemistry to proliferating and hypertrophic chondrocytes of long bone growth plates, cortical and trabecular bone osteocytes, periosteum, and articular chondrocytes (47–49). *In situ* hybridization analysis was conducted in oral tissues using a *Ddr1* probe (50). Consistent

with an epithelial pattern of expression, highest *Ddr1* mRNA levels were detected in oral epithelium including enamel organs of developing molars and basal cell layers of the oral epithelium, but low expression in ectomesenchymal tissues.

## 3.2. DDR2

Early *in situ* hybridization studies localized *Ddr2* expression to tibial growth plates (51). Subsequent more detailed analysis using $Ddr2^{+/LacZ}$ mice stained for β-galactosidase activity, first detected *Ddr2* expression in bone rudiments at E11.5 (52, 53). Analysis from E13.5 through adulthood showed strong staining in all developing skeletal elements in the appendicular, axial and cranial skeletons including growth plate cartilage, metaphyses, periosteum, cranial sutures and cranial base synchondroses. In general, expression was higher in cells representing earlier stages of each skeletal lineage. For example, in growth plates and synchondroses, expression was higher in resting and proliferating zone cells and lower in hypertrophic layers. Also, while *Ddr2* was detected in marrow and periosteal/preosteoblast layers near forming trabecular and cortical bone surfaces, no expression was detected in osteocytes. Similar periosteal localization was reported using immunohistochemistry where DDR2 colocalized with alkaline phosphatase, a preosteoblast marker (54). Notably, this distribution is very different from most of the collagen-binding integrins (α1β1, α2β1, α11β1) that are broadly expressed in connective tissues [reviewed in ref (55)]. However, there may be some overlap with integrin α10β1 which shows preferential expression in chondrocytes (11, 56). $Ddr2^{+/LacZ}$ mice were also used to examine *Ddr2* expression during tooth development (57) and in the temporomandibular joint (TMJ) (58). *Ddr2* was widely expressed in non-epithelial tooth structures including dental follicle and dental papilla during development and odontoblasts, alveolar bone osteoblast and periodontal ligament fibroblasts of adults. In contrast to the *Ddr1* mRNA distribution described above, it was conspicuously absent from epithelial structures including ameloblasts and Hertwig's epithelial root sheath. Strong *Ddr2* expression was also detected in the TMJ articular surface of adult mice. Interestingly, at this age, *Ddr2* expression in the articular surface of the knee joint was quite low suggesting differences between the fibrocartilage of the TMJ and hyaline cartilage of the knee (58).

## 3.3. Localization of DDR2 in skeletal progenitor cells

To gain further insight into the lineage of *Ddr2*-expressing cells, $Ddr2^{mer-icre-mer}$; $ROSA26^{LSLtdTomato}$ mice were developed (52, 53). After tamoxifen-induced recombination, *Ddr2*-expressing cells are labelled with tdTomato fluorescent protein, thereby allowing these cells to be followed over time. Mice were injected with tamoxifen from P1-P4 and *tdTomato*+ cells were lineage-traced for up to 2 months. Initially, *tdTomato*+cells had a similar distribution to that seen in $Ddr2^{+/LacZ}$ mice with labelling in growth plate and synchondrosis resting zone, cranial sutures, perichondrium, trabeculae, and periosteum, but absent in more differentiated cells. Over time, $tdTomato^{+}$ cells appeared in proliferating and hypertrophic chondrocytes, osteoblasts and, eventually, osteocytes. Osteoclasts were not labelled. This result is what would be expected if *Ddr2* was expressed in skeletal progenitor cells (SPCs) whose progeny became the mature cells of each skeletal lineage (hypertrophic chondrocytes for the cartilage lineage, osteocytes for the osteoblast lineage). Consistent with this concept, a high degree of colocalization between DDR2 and the skeletal progentitor/stem cell marker, GLI1 (59, 60), was observed by immunofluorescence in cranial sutures, synchondroses and tibial growth plates (52, 53). Also, $CD140\alpha^{+}/CD51^{+}$ SPCs purified from bone marrow by FACS were enriched in *Ddr2* mRNA (52).

Further evidence for DDR2 being a marker for skeletal stem cells comes from a recent study published in preprint form where DDR2 was detected in a unique cranial suture cell population (61) that could be distinguished from previously described CTSK+ suture stem cells (SSCs) (62). These $DDR2^{+}$ cells have several stem cell properties including long cycling time, capacity for self-renewal after *in vivo* implantation, potential to differentiate to osteoblasts, adipocytes and chondrocytes, expression of several SC markers including GLI1 and capacity to generate all DDR2+ cells present in the native suture. Interestingly, conditional ablation of *Ctsk*-labeled SSCs using diphtheria toxin administration to *iDTR*; *Ctsk-Cre* mice led to increased expansion of $DDR2^{+}$ suture cells and suture fusion via an endochondral mechanism. The authors postulate that $DDR2^{+}$ suture stem cells contribute to a novel form of endochondral ossification without hematopoietic recruitment; a third potential mechanism of bone formation.

## 3.4. Regulation of *Ddr2* transcription

The transcriptional control mechanisms regulating DDR2 levels in bone cells are not well understood. To date they have only been examined in cell culture where *Ddr2* is upregulated during osteoblast differentiation (63–65). One possible factor controlling this upregulation is ATF4 which, together with C/EBPβ, interacts with a C/EBP binding site at −1,150 bp in the *Ddr2* promoter to stimulate *Ddr2* expression and subsequent increases in osteoblast marker mRNAS (65). However, it is not known if these control mechanisms function *in vivo* or if other factors participate in this regulation.

# 4. Genetic models for understanding DDR functions in mineralized tissues

Experiments of nature (i.e., human genetic diseases) as well as gene inactivation mouse models have been described that, taken together, provide considerable insight into how DDRs function in bone, cartilage and the dentition.

## 4.1. Human loss-of-function mutations in *DDR2* are associated with severe skeletal and craniofacial defects while gain-of-function mutations cause fibrosis and skull abnormalities

To date, no human mutations in *DDR1* have been identified. In contrast, genetic disorders have been described associated with both loss and gain-of-function mutations in *DDR2*. Spondylo-meta-epiphyseal dysplasia with short limbs and abnormal calcifications (SMED, SL-AC) is a rare autosomal recessive genetic disorder first described in 1993 that is associated with dwarfism, short limbs, reduced bone mass, abnormal skull shape including mid-face hypoplasia and hypertelorism, open fontanelles, micrognathia and tooth abnormalities (66). This disorder was subsequently mapped to chromosome 1q23, the locus of *DDR2*, and shown to be caused by loss-of-function mutations in the DDR2 tyrosine kinase domain as well as mutations affecting intracellular trafficking (67–70). Unfortunately, individuals with this disorder rarely survive beyond childhood; atlantoaxial instability and resulting spinal cord damage is the most common cause of death (71, 72). The short lifespan of SMED, SL-AC patients compounded with the rarety of this disorder have limited studies in humans.

A second disorder, designated as Warburg-Cinotti Syndrome, was described in 2018 and associated with putative activating mutations in the DDR2 kinase domain (73). Fibroblasts from patients exhibited high levels of DDR2 phosphorylation in the absence of collagen stimulation, suggesting that receptor activation was ligand-independent. This disorder, which is inherited in an autosomal dominant manner, is associated with progressive fibrosis, corneal vascularization, skull abnormalities and osteolysis. In view of the deleterious effects of *DDR2* loss-of-function mutations on bone formation in SMED, SL-AC pateints, it is not clear why activating mutations would lead to an osteolytic phenotype. However, since only 6 patients with Warburg-Cinotti Syndrome have been described, the phenotypic variation within this disorder cannot be currently assessed.

*DDR2* may also be a determinant of bone mineral density (BMD) and fracture risk in human populations. Analysis of a Chinese Han population and an American Caucasian population identified 28 SNPs in *DDR2*. Of these, 3 were significantly associated with hip BMD in the Chinese, but not American population (74). Although this preliminary finding suggests that certain polymorphisms in *DDR2* may be risk factors for osteoporosis, more studies are needed, particularly in diverse populations to assess the significance of these findings.

As will be described below, the phenotypic similarities between SMED, SL-AC patients and *Ddr2*-deficient mice indicate that mouse models are an appropriate model for studying this disease.

## 4.2. Global *Ddr1* and *Ddr2* knockout models suggest roles in bone and tooth development

As shown in early studies, global knockout of either *Ddr1* or *Ddr2* resulted in dwarf phenotypes, particularly for *Ddr2*-deficient mice (46, 51). However, different bases for the observed growth deficits were proposed. In *Ddr1* deficient mice, all organs were proportionally smaller suggesting an overall growth defect (46). However, no differences in growth plate size, chondrocyte proliferation or apoptosis were noted.

In contrast, initial analysis of globally *Ddr2* deficient mice showed prominent growth retardation that was attributed to decreased proliferation of growth plate chondrocytes in the absence of changes in apoptosis resulting in shortened growth plates (51). A similar phenotype was subsequently observed in $Ddr2^{slie/slie}$ mice, which have a spontaneous 150 kb deletion in *Ddr2* that encompasses exons 1–17 to produce an effective null allele (75). A more detailed analysis of the bone phenotype of $Ddr2^{slie/slie}$ mice revealed that skeletal growth defects were accompanied by large reductions in trabecular bone volume, trabecular thickness and number, changes that were attributed to reduced bone formation rate rather than stimulation of osteoclastic bone resorption (65). Similar changes in vertebral trabecular bone were also seen. However, cortical bone was only slightly affected. Interestingly, the reduction in bone mass with *Ddr2* deficiency was accompanied by an increase in marrow fat. Consistent with these changes, bone marrow stromal cells (BMSCs) or calvarial preosteoblasts cultured from $Ddr2^{slie/slie}$ mice exhibited defective osteoblast differentiation while differentiation of BMSCs to adipocytes was enhanced.

Changes in craniofacial morphology in *Ddr1* and *Ddr2*-deficient mice have been compared using a machine learning approach that was able to clearly discriminate between skulls from wildtype, *Ddr1* and *Ddr2*-deficient mice (76). Although *Ddr1*-deficient skulls are somewhat smaller than wild type controls, they have no substantial alterations in relative skull dimensions. In contrast, skulls from *Ddr2*-deficient mice are dramatically shorter in the anterior-posterior direction with a more spherical skull shape associated with increased anterior skull width as well as reduced nasal bone length. Subsequent analysis of this phenotype identified a defect in proliferation of synchondrosis chondrocytes, particularly in the intersphenoid synchondrosis, in the absence of changes in apoptosis (53). These changes were associated with a characteristic expansion of the synchondrosis resting zone, possibly related to the defective conversion of these cells into proliferating chondrocytes. *Ddr2*-deficient skulls also have open fontanelles at birth, thinning of frontal bones and defects in frontal suture fusion that persist into adulthood (53, 65).

Effects of global *Ddr1* and *Ddr2* inactivation on the dentition were also examined. *Ddr1*-deficient mice had normal teeth, but age-dependent periodontal degeneration including alveolar bone loss was noted (50). In contrast, teeth from $Ddr2^{slie/slie}$ mice had

smaller roots and reduced crown/root ratio resulting in disproportionate tooth size (57). These mice also exhibited gradual alveolar bone loss over a 10-month period due to increased osteoclast activity as well as atypical periodontal ligament collagen fibrils.

## 4.3. Conditional *Ddr1* and *Ddr2* inactivation studies in bone

In addition to affecting the skeleton, global *Ddr1* deficiency inhibits uterine development and embryo implantation as well as mammary epithelium development leading to defective milk production (46). Likewise, *Ddr2* deficiency reduces fertility by inhibiting female and male gonadal function and steroid hormone production leading to partial sterility and interferes with certain metabolic activities (75) (see **Section 8**). Because effects of global inactivation of *Ddr1* or *Ddr2* are not restricted to the skeleton, specific cell-autonomous functions of these collagen receptors in bone cannot be inferred from global knockout studies. Although several early studies with osteoblast and chondrocyte cell lines and primary cultures suggested direct functions for DDR1 and 2 in bone cells (48, 63, 64), this issue was not resolved until recently when results of tissue-specific *Ddr1* and *Ddr2* knockouts were reported.

### 4.3.1. *Ddr1*

Chondrocyte or osteoblast-selective inactivation of *Ddr1* was achieved by crossing $Ddr1^{fl/fl}$ mice with $Col2a1^{CreERT}$ or $Col1a1^{CreERT}$ mice (47–49). Chondrocyte-selective knockout of *Ddr1* in tamoxifen-treated $Col2a1^{CreERT}$; $Ddr1^{fl/fl}$ mice led to a 10–20 percent decrease in body weight and length and delayed formation of a secondary ossification center (47). In contrast to early reports with global *Ddr1* knockouts (46), decreases in chondrocyte proliferation, apoptosis and hypertrophy were reported (47). These changes were accompanied by an approximately 20 percent change in trabecular bone volume while cortical thickness was unchanged. In addition, the chondrocyte hypertrophy markers (ColX, MMP13, RUNX2) and hedgehog pathway intermediate, IHH, all decreased. These results suggest that inactivation of *Ddr1* in chondrocytes preferentially affects endochondral ossification. Results with $Col1a1^{CreERT}$; $Ddr1^{fl/fl}$ mice, where *Ddr1* was preferentially inactivated in osteoblasts/osteocytes were markedly different from chondrocyte-selective knockouts (48). In this case, minimal changes in endochondral ossification or trabecular bone parameters were noted while cortical thickness was reduced by approximately 50 percent. These changes were accompanied by a loss of mechanical properties and inhibition of osteoblast markers such as RUNX2, ALPL, BGLAP and COLIA1. In a second study with $Col1a1^{CreERT}$; $Ddr1^{fl/fl}$ mice, the same group examined the consequences of *Ddr1* inactivation in adults over extended periods (49). In this case, modest changes in trabecular parameters were noted together with reductions in cortical thickness, osteoblast differentiation markers and cortical bone formation rate. These changes were accompanied by increased apoptosis and autophagy markers. No craniofacial changes were described in any of these studies.

### 4.3.2. *Ddr2*

Conditional knockout studies with *Ddr2* were informed by results of localization and lineage tracing experiments showing preferential expression of this gene in GLI1+ skeletal progenitor cells, chondrocytes, and osteoblasts (see **Sections 3.2**, **3.3**). To determine functions of *Ddr2* in these cells, $Ddr2^{fl/fl}$ mice were crossed with $Gli1^{CreERT}$, $Col2a1^{Cre}$or $Bglap^{Cre}$ mice and resulting long bone and craniofacial phenotypes examined (52, 53). Inactivation of *Ddr2* in *Gli1*-expressing cells, induced by injecting neonatal $Gli1^{CreERT}$; $Ddr2^{fl/fl}$ mice with tamoxifen, resulted in essentially the same phenotype seen in $Ddr2^{slie/slie}$ mice. Dwarfism was observed in both males and females, and this was associated with an approximately 12 percent reduction of growth plate length at P14. In addition, severe defects in endochondral bone formation were observed, particularly in males where trabecular BV/TV was reduced by approximately 50 percent. Associated reductions in trabecular number and thickness and increased trabecular spacing were also seen at 3 months. However, cortical BV/TV was not affected. The craniofacial phenotype of $Gli1^{CreERT}$; $Ddr2^{fl/fl}$ mice was also essentially identical to $Ddr2^{slie/slie}$ mice; anterior-posterior skull length was reduced with an associated increase in anterior skull width. Mutants also exhibited frontal bone thinning and shortened nasal bones (53). Also like global knockouts, the anterior portion of frontal sutures failed to mineralize in most mice.

The phenotype of $Col2a1^{Cre}$; $Ddr2^{fl/fl}$ mice was similar to $Gli1^{CreERT}$; $Ddr2^{fl/fl}$ and $Ddr2^{slie/slie}$ mice with the important exception that no defects in suture fusion were observed. Although it has been proposed that changes in growth of the cranial base can affect suture fusion (77), this is clearly not an adequate explanation for effects of *Ddr2* inactivation on frontal sutures since $Col2a1^{Cre}$; $Ddr2^{fl/fl}$ mice had the same cranial base growth defects seen in $Gli1^{CreERT}$; $Ddr2^{fl/fl}$ mice. Based on this result, it was concluded that functions of *Ddr2* in synchondrosis endochondral bone formation are independent from its functions in cranial sutures. Consistent with the observed reduction in tibial bone formation, mRNA levels of osteoblast and hypertrophic chondrocyte markers were all reduced in $Col2a1^{Cre}$; $Ddr2^{fl/fl}$ mice. These changes were accompanied by decreased mRNA levels of the hedgehog pathway intermediates, *Ihh* and *Gli1*. Since defects in Hh signaling were also noted with conditional *Ddr1* knockout (47) (Section 4.3.1), this pathway may be a common target for DDRs.

Although *Ddr2* was expressed in osteoblasts on trabecular and periosteal surfaces, it probably does not have a major function in mature osteoblasts since $Bglap^{Cre}$; $Ddr2^{fl/fl}$ mice were essentially identical to wild type control mice. Because this Cre is mainly active in mature osteoblasts and, possibly, osteocytes, it is still possible that *Ddr2* may have functions in earlier stages of the osteoblast lineage.

Overall, *Ddr2* conditional knockout studies support the concept that this gene functions in earlier stages of bone formation (i.e., in $Gli1^{CreERT}$-positive skeletal progenitor cells and

*Col2a1*$^{Cre}$-positive resting zone and proliferative chondrocytes) rather than in terminally differentiated osteoblasts or hypertrophic chondrocytes. Two cell culture studies reinforce this conclusion (52). In the first, E12.5 limb buds from *Ddr2*$^{fl/fl}$ mice were used to prepare micromass cultures enriched in chondro-osteo progenitors that were treated with control or Cre adenovirus before growth in chondrogenic medium. *Ddr2* inactivation strongly inhibited chondrogenesis as measured by Alcian blue staining or expression of chondrocyte markers. In the second study, CD140α$^{+}$/CD51$^{+}$ SPCs were prepared from *Ddr2*$^{fl/fl}$ mice and grown in osteogenic medium after treatment with Cre adenovirus. In this case, *Ddr2* inactivation strongly inhibited osteoblast differentiation (mineralization and expression of osteoblast markers).

### 4.3.3. Possible functions of *Ddr2* in osteoclasts

The studies described above all focused on functions of *Ddr2* in chondro-osteo lineage cells which form chondrocytes, osteoblasts and osteocytes. However, there is still some controversy regarding possible *Ddr2* functions in osteoclasts. On one hand, lineage tracing studies with *Ddr2*$^{mer-icre-mer}$; *ROSA26*$^{LSLtdTomato}$ mice did not show colocalization of the tdTomato label with TRAP-positive osteoclasts (**Section 3.3**) and globally *Ddr2* deficient mice (*Ddr2*$^{slie/slie}$ mice) did not have any detectable changes in bone resorption markers or osteoclast differentiation capacity (**Section 4.1**). On the other hand, evidence was presented that DDR2 has a suppressive effect on osteoclast formation in cell culture models (78). DDR2 protein and mRNA were detected at low levels in the RAW264.7 macrophage cell line and primary cultures of bone marrow macrophage and these levels were further reduced with *in vitro* induction of osteoclast formation. Also, overexpression of *Ddr2* in RAW264.7 was shown to inhibit osteoclast induction while shRNA knockdown of *Ddr2* further stimulated this process. Furthermore, adenovirus-mediated overexpression of *Ddr2* in the femur marrow cavity partially reversed osteoporosis in ovariectomized mice, a phenotype that is largely due to osteoclast activation. These studies suggest that *Ddr2* can function in the monocytic lineage to suppress osteoclastogenesis. Lastly, in a recent study *Ddr2*$^{fl/fl}$ mice were crossed with *LysM*$^{Cre}$ mice to conditionally inactivate *Ddr2* in myeloid lineage cells (79). The resulting animals had a hyperinflammatory phenotype after exposure to either collagen antibody-induced arthritis or a high-fat diet. After arthritis induction, mice had increased ankle inflammation, elevation of inflammatory markers, increased bone resorption and increased osteoclast surface per bone surface as well as an approximately 15 percent decrease in bone mineral density. Also, evidence was presented that loss of DDR2 increased macrophage repolarization from an M2 to M1 phenotype resulting in enhanced inflammation. However, this study did not look for changes in bone density in the absence of an inflammatory stimulus. Nevertheless, this work supports a role for DDR2 in the suppression of osteoclastogenesis through its inhibitory actions on monocytic osteoclast precursors. However, it is still not clear why, in previous studies, changes in bone resorption markers were not detected in *Ddr2*$^{slie/slie}$ mice or why osteoclasts were not detected as part of the DDR2 lineage (52, 65). It is possible that effects on bone resorption in the absence of induced inflammation may not be large enough to affect bone mass or, alternatively, that in globally *Ddr2* deficient mice, interference with other DDR2 dependent processes may compensate for effects on osteoclastogenesis. Another possibility would be that DDR2 is not expressed in the osteoclast lineage and does not have a direct function in these cells, but rather modulates effects of macrophage on osteoclastogenesis. Studies where *Ddr2* is more selectively inactivated only in osteoclasts (for example, using *Ctsk-Cre* or *TRAP-Cre*) may be necessary to resolve this issue (80).

## 4.4. DDR2-dependent changes in osteoblast gene expression

A consistent finding from *Ddr2* knockout studies is that osteoblast differentiation and associated expression of osteoblast marker genes is suppressed. A limited number of studies have investigated the basis for this suppression. Because of its central role as a master transcriptional regulator of bone formation, studies to date have focused on RUNX2. This transcription factor is expressed at early times during bone development coincident with the formation of cartilage condensations and has roles in both hypertrophic cartilage formation as well as osteoblast differentiation [for review (81)]. RUNX2 activity is subject to several controls including phosphorylation by ERK1/2 and p38 mitogen-activated protein kinases (MAPKs) (82). Both MAPKs are important for bone formation as demonstrated by *in vivo* gain and loss-of-function studies (83, 84). Once activated, MAPKs translocate to the nucleus where they bind and phosphorylate RUNX2 on the chromatin of target genes (85). MAPKs phosphorylate RUNX2 on several serine residues, the most important being Ser301 and Ser319 (86). Phosphorylated RUNX2 recruits specific histone acetyltransferases and methylases to chromatin resulting in increased H3K9 and H4K5 acetylation and H3K4 di-methylation, histone modifications associated with transcriptional activation, as well as decreased H3K9 mono-, di and tri-methylation, histone marks associated with repression (85). These changes open chromatin structure to allow RNA polymerase II to bind and initiate transcription of osteoblast-related genes. RUNX2 phosphorylation and MAPK activity are obligatory for these changes since transfection of cells with a phosphorylation-resistant S301,319A mutant RUNX2 or treatment with MAPK inhibitors blocks transcription.

Since both ERK1/2 and p38 MAPKs are known downstream responses to DDR2 activation (25), it was hypothesized that this pathway could explain the observed stimulatory effects of DDR2 on osteoblast gene expression. This concept has been tested in cell culture studies with osteoblast cell lines as well as in osteoblasts from *Ddr2*-deficient mice (64, 65). In early studies with osteoblast cell lines and primary BMSC cultures, DDR2 was shown to stimulate osteoblast differentiation through a pathway involving ERK/MAPK activation and RUNX2 phosphorylation (64). *Ddr2* shRNA inhibited differentiation while overexpression was stimulatory. These changes were paralleled, respectively, by

increased or decreased ERK/MAPK activity, RUNX2 phosphorylation and transcriptional activity. Significantly, effects of *Ddr2* shRNA knockdown could be overcome by transfecting cells with a phosphomimetic Runx2 S301,319E mutant where replacement of alanine with glutamate mimics a phosphate group. In separate studies referenced in Section 4.1 (65), calvarial preosteoblasts or BMSCs isolated from *Ddr2*$^{slie/slie}$ mice were found to be deficient in ability to undergo osteoblast differentiation while BMSCs from these mice exhibited enhance adipogenic differentiation. The reduced osteoblast differentiation in *Ddr2*-deficient cells was directly related to reduced ERK/MAPK activity and RUNX2-S319 phosphorylation and was rescued by transfection with the RUNX2 S301/319E mutant described above. The ability of DDR2 to stimulate ERK/MAPK activity may also explain the increase in marrow fat observed in *Ddr2*$^{slie/slie}$ mice. In addition to phosphorylating RUNX2, ERK1/2 can phosphorylate the adipogenic transcription factor, PPARγ, on Ser112. In this case, however, phosphorylation inhibits transcriptional activity. By preventing this inhibitory phosphorylation, *Ddr2* knockout would be expected to restore PPARγ activity to permit formation of marrow fat. Consistent with this interpretation, transgenic mice containing a phosphorylation-resistant S112A PPARγ mutant have increased marrow fat and reduced bone mass (87).

## 5. Requirement for DDR2 in bone regeneration

Consistent with the marked effects of *Ddr2* deficiency on bone development, inactivation of this gene was also shown to inhibit bone regeneration. Two regeneration models were examined, a calvarial bone defect and a tibial fracture (88, 89). For the calvarial model, a 0.5 mm burr hole defect was generated in wild type or *Ddr2*$^{slie/slie}$ mice and regeneration was examined for increasing times up to 12 weeks. In wild type mice, this type of defect was completely healed after 4 weeks while no bone bridging was seen in mutant mice even after 12 weeks. *Ddr2*, which was expressed in sutures and periosteal cells before injury, was detected in the injury site within 3 days and expanded during the healing process. Also, inactivation of *Ddr2* in calvarial cells in culture reduced osteoblast differentiation. For the fracture model, a mid-shaft tibial fracture was created in wild type or *Ddr2*$^{slie/slie}$ mice and fracture healing was monitored for 3 weeks. In this case, *Ddr2*-deficient mice were unable to form complete unions at the fracture site as measured by Radiographic Union Score Tibia (mRUST) (90).

## 6. Functions of DDR2 in cartilage matrix organization and relationship to ECM stiffness

In the studies described above, the reduced linear growth of long bones and skulls in *Ddr2*-deficient mice was attributed to proliferation defects in growth plate and synchondrosis chondrocytes in the absence of changes in apoptosis (52, 53). Interestingly, an examination of chondrocyte morphology revealed that the normal organization of these cells into columns was disrupted with *Ddr2* inactivation. This effect was seen in long bone growth plates but was particularly striking in cranial base synchondroses where the central resting zone was greatly expanded with widely separated disorganized cells (52, 53). In some cases, chondrocytes actually shifted their orientation by 90 degrees to form an ectopic hypertrophic zone at right angles to the normal plane of synchondrosis organization. These changes were accompanied by loss of chondrocyte polarity as measured by disruption of the normally consistent orientation of GM130, a Golgi apparatus marker, relative to the nucleus and anterior-posterior axis of the skull. This may explain the proliferation defect seen in chondrocytes of *Ddr2*-deficient mice since disruption of GM130 orientation is known to impair spindle assembly and cell division (65). The relevance of these findings to human physiology is emphasized by the observation that collagen matrix distribution is also disrupted in growth plate cartilage from SMED, SL-AC patients (66).

How might DDR2 affect chondrocyte polarity? One possibility is that it is necessary for collagen matrix organization and fibril orientation which would subsequently affect chondrocyte orientation. Examination of the type II collagen distribution in both growth plates and synchondroses by immunofluorescence microscopy revealed a shift from a uniform distribution in the territorial matrix next to chondrocytes and the extraterritorial matrix between cell clusters in wild type mice to an uneven distribution restricted to the pericellular space adjacent to chondrocytes in mutants (53). These changes were accompanied by loss of type II collagen fibril orientation as measured by second harmonic generation (SHG) microscopy. This analysis detected a dramatic shift from a highly oriented matrix (high anisotrophy) in synchondroses of wild type mice to a disorganized matrix (low anisotropy) in mutants where fibrils had a randomized orientation (53). Although primary cilia have been related to cell polarity and collagen orientation in other systems (91), regulation of this important organelle by DDR2 has not been reported.

Another consequence of DDR2 maintaining collagen fibril orientation is an increase in overall ECM stiffness. Although this has not been examined during bone development, there are several examples in other experimental systems. For example, DDR2 in breast cancer-associated fibroblasts (CAFs) increases tumor stiffness by organizing type I collagen fibrils (92). Also, at sites of trauma-induced heterotopic ossification, DDR2 increases collagen fibril orientation as measured by SHG (93) (also see Section 7.3). In both cases, evidence was presented that DDR2 functioned in concert with collagen-binding β1 integrins to stimulate, on one hand, tumor metastasis to the lungs or, on the other, ectopic bone formation. As noted in Section 2, fibrillar collagens I–III contain binding sites for both DDRs and integrins always separated by 96 amino acid residues. This characteristic spacing may allow collagen to simultaneously regulate both these receptors. For example, in breast tumor metastasis, DDR2 was found to stimulate CAF-mediated mechanotransduction by increasing integrin activation in response to collagen. This was accomplished by stimulating RAP1-mediated Talin1 and Kindlin2 recruitment to integrins in focal adhesions (92). Also, in trauma-

induced heterotopic ossification, DDR2 was necessary for full activation of integrin-dependent signals such as focal adhesion kinase (FAK) activation as well as nuclear levels of the Hippo pathway intermediate, TAZ, and its downstream targets (93).

## 7. Involvement of DDRs in abnormal ossification

Given the involvement of DDRs in normal bone formation, it is not totally surprising that they are also involved when this process goes awry. It this section, DDR involvement in vascular calcification, osteoarthritis and heterotopic ossification will be discussed.

### 7.1. Vascular calcification

Initiated by insults such as high levels of circulating LDL cholesterol, diabetes or chronic kidney disease, vascular calcification is a key event in advanced atherosclerosis. Calcium phosphate crystals can be deposited either in the subepithelial intima of blood vessels (intimal calcification) or in the smooth muscle-rich media (medial calcification) (94). This latter process shares many similarities with normal bone formation. It is initiated by differentiation of vascular smooth muscle cells or SMC progenitors into osteochondroprogenitor cells which form bone-like structures in arteries through a process that mimics endochondral bone formation as indicated by formation of cartilage that subsequently is converted into a bone-like structure (95). Like normal bone formation, this process requires interactions of progenitor cells with type I collagen and is mediated by the master transcriptional regulator of bone formation, RUNX2 (96, 97). Vascular calcification can be induced in mice by feeding LDL receptor-deficient animals ($Ldlr^{-/-}$ mice) a high fat, high cholesterol diet. Breeding a *Ddr1*-null allele into $Ldlr^{-/-}$ mice resulted in animals that were resistant to developing vascular calcification (97). Subsequent analysis showed that calcification was inhibited via a mechanism involving suppression of phosphatidyl inositol-3-kinase/AKT and p38/ERK MAP kinase signaling and inhibition of RUNX2 phosphorylation and activation (98). More recent studies extended this work by showing that DDR1 up-regulates its own synthesis in response to the stiffness of the matrix environment around VSMCs. This is accomplished by stimulating the nuclear translocation of the Hippo pathway intermediates, YAP and TAZ, to increase *Ddr1* transcription and subsequent mineralization (99). This may explain the known relationship between arterial stiffening and acceleration of vascular calcification (100).

### 7.2. Osteoarthritis

Osteoarthritis (OA), a primary indicator for joint degeneration, is characterized by cartilage degradation, osteophyte formation and joint mineralization (101). OA can occur in fibrocartilage of the temporomandibular joint (TMJ) or in hyaline cartilage of major joints such as the knee. OA in hyaline cartilage generally increases with age. In contrast, TMJ OA has an earlier onset (102, 103). Interactions between chondrocytes and the ECM of hyaline cartilage and fibrocartilage may be key factors for understanding OA pathogenesis in these two tissues. TMJ fibrocartilage extracellular matrix mainly contains type I collagen while type II collagen predominates in hyaline joints (104). Both DDR1 and DDR2 are involved in OA etiology although they may function through different mechanisms. Unlike DDR1, which is broadly but weakly activated by collagens I to IV, DDR2 is strongly activated by types I and III collagen of TMJ fibrocartilage but is less responsive to type II collagen (28). *Ddr2* is expressed at low levels in healthy adult hyaline cartilage joints but is abundant in TMJ fibrocartilage (58). Thus, *Ddr2* is normally expressed at highest levels in an ECM environment that is conducive to its activation. Consistent with its distribution, *Ddr2* is required for normal TMJ formation; global *Ddr2* inactivation disrupts TMJ development beginning in neonates which show an initial delay in condyle mineralization that persist in adults leading to eventual joint degeneration and subchondral bone loss (58). In contrast, knee joints, which are composed of hyaline cartilage, are not affected by *Ddr2* deficiency. *Ddr1* global knockout mice, in contrast, exhibit a spontaneous rapid-onset TMJ OA that is seen by 9 weeks without involvement of other joints (105). The authors of this study proposed that induction of TMJ OA is related to the observation that loss of DDR1 was accompanied by a compensatory up-regulation of DDR2. This is then activated by the type I collagen in TMJ fibrocartilage to induce OA. It is not known if these changes are seen in *Ddr1*-deficient neonates although a separate study reported TMJ abnormalities in mice as young as 4 weeks (50).

DDR2 has also been related to OA in hyaline cartilage joints. In this case, the normally low levels of DDR2 in adults are increased with injuries such as trauma or surgical destabilization of the medial meniscus, which subsequently induce OA (106). In this case, globally *Ddr2*-deficient mice or mice where *Ddr2* in selectively inactivated in articular cartilage are resistant to surgically-induced OA indicating that DDR2 is required for OA induction in this tissue (107, 108). However, overexpressing *Ddr2* in hyaline cartilage does not lead to spontaneous OA formation unless hyaline cartilage ECM is altered by trauma (106, 108). It has been proposed that trauma-induced damage to the ECM may disrupt the pericellular matrix around chondrocytes and allow them to interact with type II collagen fibrils resulting in DDR2 activation and OA (107).

### 7.3. Heterotopic ossification

Heterotopic ossification (HO) is a debilitating condition that occurs after many traumatic injuries. In HO, PDGFRα+ connective tissue cells present in soft tissue adjacent to the injury site change their differentiation trajectory to form ectopic cartilage and bone (109). *Ddr2* has been recently shown to play a role in the pathogenesis of HO (93). Using single cell RNA sequencing, *Ddr2* was discovered to be highly expressed by

PDGFRα+ cells, that form the major cell lineages involved in HO formation. In HO, both DDR2 and phospho-DDR2, a marker of active DDR2, were shown to be significantly upregulated in PDGFRα+ cells within the tendon, peritendon, and soft tissue areas surrounding the HO site. Interestingly, DDR2 mediates HO formation after injury, as both *Ddr2*$^{slie/slie}$ mice (global knockout) and tamoxifen-treated *Pdgfa-Cre*$^{ER}$; *Ddr2*$^{fl/fl}$ mice (conditional knockout in progenitor cells) display significant reductions in *Sox9* expressing chondrocytes, safranin O labeled cells and reductions in ectopic bone formation due to extracellular matrix disorganization and FAK/YAP/TAZ dysregulation (described in Section 5). This study highlights how extracellular matrix alignment can have profound effects on HO progression and how DDR2 is an important regulator of this process.

## 8. Metabolic effects of Ddr2 deficiency and relationship to bone metabolism

In addition to inhibiting skeletal growth, global *Ddr2* deficiency also affects metabolism. For example, *Ddr2*$^{slie/slie}$ mice have elevated blood glucose levels, reduced body fat and increased lean body mass (75), elevated levels of circulating adiponectin and decreased serum leptin (65). It is not known if there is a relationship between these metabolic changes and the bone phenotype of these mice. However, as discussed in Sections 4.1, 4.3, the decrease in bone mass in *Ddr2*$^{slie/slie}$ mice is paralleled by an increase in marrow fat, a change that may be related to the reduced ERK/MAPK activity in mutant mice. The consequences of this reduced MAPK activity would include suppression of RUNX2 and PPARγ phosphorylation, decreased osteoblast and increased marrow adipocyte gene expression and differentiation. Since marrow adipocytes are a major source of serum adiponectin (110), the increase in marrow adipocytes in *Ddr2*$^{slie/slie}$ mice may explain the observed increase in serum adiponectin. However, specific knockout of the Adipoq gene in marrow adipocytes using a recently described double recombination strategy (111) would be necessary to definitively test this hypothesis.

Interestingly, *Ddr2* is expressed in adipocytes. Early studies suggested possible direct effects of DDR2 on these cells such as suppression of insulin stimulated tyrosine phosphorylation of the insulin receptor in the 3T3-L1 adipocyte cell line (112). More recently, direct effects of DDR2 on adipocytes *in vivo* were examined using *Adipo*$^{Cre}$; *Ddr2*$^{fl/fl}$ mice, where *Ddr2* is inactivated in peripheral as well as marrow fat (113). In this study, mutant mice were protected from high fat diet-induced weight gain, a response that was attributed to decreased adipocyte size. Significantly, these animals also had a high bone mass phenotype accompanied by increases in both bone formation rate and resorption. These changes were explained by a DDR2-specific repression of adenylate cyclase 5 (Adcy5) in adipocytes that is removed in mutant mice leading to increased cAMP production and lipolysis in marrow adipocytes. The released fatty acids in the marrow cavity then promote increased oxidative metabolism in osteoblast leading to increased osteoblast and osteoclast activity. Therefore, by modulating lipolysis in adipocytes, DDR2 can indirectly control bone formation. This mechanism may complement the more direct effects of DDR2 on skeletal progenitor cells described in Section 4.3.2.

## 9. Summary and future perspectives

The study of DDR functions in bone is a relatively new research area and many questions remain about what these collagen receptors do and how they do it. As shown in this review, both DDR1 and DDR2 have functions in mineralized tissues with DDR2 perhaps having a greater role under physiological conditions. However, clear functions for DDR1 are also seen, particularly in pathological conditions such as vascular calcification.

Although tissue distribution studies, particularly for DDR1, are incomplete, the original conclusion that DDR1 functions in epithelia while DDR2 is in connective tissues may need revision, particularly for DDR1, which has clear functions in connective tissues like cartilage and bone. More detailed DDR1 localization and lineage tracing studies will be required to more fully understand where this collagen receptor functions. The observation that DDR2 is present in GLI1-positive skeletal progenitor cells of cranial sutures and, possibly, cartilage where it controls cell proliferation and differentiation to chondrocytes and osteoblasts is of particular interest. These studies suggest that DDR2, together with collagen binding integrins, allows certain classes of skeletal progenitor/stem cells to sense their ECM environment and modulate their differentiation state according to ECM stiffness and mechanical loads. As the more ancient of the two collagen receptors, the DDRs were likely complemented by the newly emerging collagen-binding integrins when the vertebrate skeleton first evolved so that these two receptors now work in concert. Another intriguing area is the possible function of DDR2 in osteoimmunology where it may modulate activities of various myeloid lineages to control inflammation and bone resorption.

Although conditional knockout studies showed that DDR2 functions in skeletal progenitor cells and chondrocytes, little is known about its actual mechanism of action in these tissues. Current, albeit incomplete, knowledge in this area is summarized in Figure 2. Some of its activities may be explained by modulation of MAP kinases which subsequently control osteogenic and adipogenic transcription factors through phosphorylation. However, this is likely only part of the story. The dramatic effects of DDR2 on collagen fibril orientation, matrix stiffness and cell polarity may also be an important part of an overall mechanism that still needs to be discerned. By modulating matrix stiffness-associated pathways including the Hippo pathway, DDR2 and integrins may work together to control stiffness-associated nuclear changes and transcription. These matrix signals may also modify the response of cells to soluble signals coming from growth factors or morphogens. All these topics are clearly fruitful areas for future investigations.

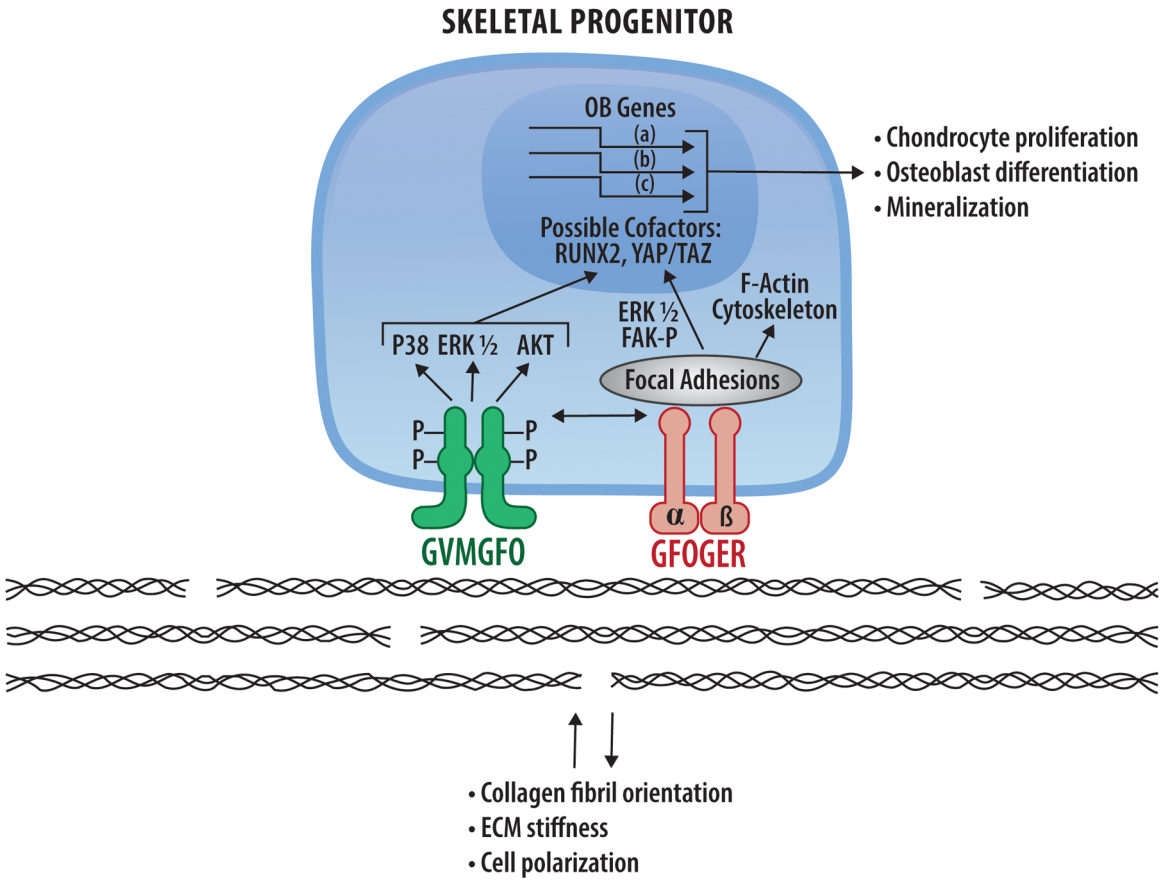



FIGURE 2
DDR2 signaling mechanisms in bone and potential interactions with collagen-binding integrins. DDR2 (green) and integrins (pink) bind and are activated by the specific sequences in fibrillar collagens indicated. Downstream signals arising from DDR2 and integrin activation are indicated as are nuclear changes in RUNX2 and HIPPO pathway intermediates, YAP and TAZ, resulting in transcriptional changes necessary for chondrocyte proliferation, osteoblast differentiation and mineralization (see Sections 4.4). Also shown are DDR-dependent changes in collagen matrix organization and cell polarity described in Section 6.

Recent discoveries on DDR function may also have important implications for the treatment of disease. For example, the demonstrated role of DDR1 in vascular calcification and of DDR2 in osteoarthritis and heterotopic ossification suggest that specific DDR2 inhibitors already under development could be used to treat these disorders (114). Also, the recent discovery that DDR2 is required for skeletal regeneration may open new directions for therapy through the development of either DDR-activating tissue engineering scaffolds or other treatments that modify DDR activity.

Clearly, the study of DDRs in bone will continue to be a growing area of musculoskeletal research that holds much promise for exciting future discoveries.

## Author contributions

RF wrote and edited the article. SH wrote portions of the article and edited the entire article CG wrote portions of the article and edited the entire article. All authors contributed to the article and approved the submitted version.

## Funding

Research from the authors laboratory described in this article was supported by NIH/NIDCR grants DE11723, DE029012, DE029465, DE030675, Department of Defense grant PR190899, research funds from the Department of Periodontics and Oral Medicine, University of Michigan School of Dentistry and the Michigan Musculoskeletal Health Core Center (NIH/NIAMS P30 AR069620).

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated

organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

## References


1. Franceschi RT, Iyer BS. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. *J Bone Miner Res.* (1992) 7(2):235–46. doi: 10.1002/jbmr.5650070216

2. Franceschi RT. The developmental control of osteoblast-specific gene expression: role of specific transcription factors and the extracellular matrix environment. *Crit Rev Oral Biol Med.* (1999) 10(1):40–57. doi: 10.1177/10454411990100010201

3. Lynch MP, Stein JL, Stein GS, Lian JB. The influence of type I collagen on the development and maintenance of the osteoblast phenotype in primary and passaged rat calvarial osteoblasts: modification of expression of genes supporting cell growth, adhesion, and extracellular matrix mineralization. *Exp Cell Res.* (1995) 216 (1):35–45. doi: 10.1006/excr.1995.1005

4. D'Alonzo RC, Kowalski AJ, Denhardt DT, Nickols GA, Partridge NC. Regulation of collagenase-3 and osteocalcin gene expression by collagen and osteopontin in differentiating MC3T3-E1 cells. *J Biol Chem.* (2002) 277(27):24788–98. doi: 10.1074/jbc.M202604200

5. Togari A, Arai M, Nakagawa S, Banno A, Aoki M, Matsumoto S. Alteration of bone status with ascorbic acid deficiency in ODS (osteogenic disorder shionogi) rats. *Jpn J Pharmacol.* (1995) 68(3):255–61. doi: 10.1254/jjp.68.255

6. Mahmoodian F, Gosiewska A, Peterkofsky B. Regulation and properties of bone alkaline phosphatase during vitamin C deficiency in Guinea pigs. *Arch Biochem Biophys.* (1996) 336(1):86–96. doi: 10.1006/abbi.1996.0535

7. Fedarko NS, Moerike M, Brenner R, Robey PB. Extracellular matrix formation by osteoblasts from patients with osteogenesis imperfecta. *J Bone Miner Res.* (1992) 7 (8):921–30. doi: 10.1002/jbmr.5650070809

8. Fedarko NS, Sponseller PD, Shapiro JR. Long-term extracellular matrix metabolism by cultured human osteogenesis imperfecta osteoblasts. *J Bone Miner Res.* (1996) 11(6):800–5. doi: 10.1002/jbmr.5650110611

9. Kechagia JZ, Ivaska J, Roca-Cusachs P. Integrins as biomechanical sensors of the microenvironment. *Nat Rev Mol Cell Biol.* (2019) 20(8):457–73. doi: 10.1038/s41580-019-0134-2

10. Saito T, Albelda SM, Brighton CT. Identification of integrin receptors on cultured human bone cells. *J Orthop Res.* (1994) 12(3):384–94. doi: 10.1002/jor.1100120311

11. Lundgren-Akerlund E, Aszodi A. Integrin alpha10beta1: a collagen receptor critical in skeletal development. *Adv Exp Med Biol.* (2014) 819:61–71. doi: 10.1007/978-94-017-9153-3_4

12. Xiao G, Wang D, Benson MD, Karsenty G, Franceschi RT. Role of the alpha2-integrin in osteoblast-specific gene expression and activation of the Osf2 transcription factor. *J Biol Chem.* (1998) 273(49):32988–94. doi: 10.1074/jbc.273.49.32988

13. Brunner M, Jurdic P, Tuckerman JP, Block MR, Bouvard D. New insights into adhesion signaling in bone formation. *Int Rev Cell Mol Biol.* (2013) 305:1–68. doi: 10.1016/B978-0-12-407695-2.00001-9

14. Ekholm E, Hankenson KD, Uusitalo H, Hiltunen A, Gardner H, Heino J, et al. Diminished callus size and cartilage synthesis in alpha 1 beta 1 integrin-deficient mice during bone fracture healing. *Am J Pathol.* (2002) 160(5):1779–85. doi: 10.1016/S0002-9440(10)61124-8

15. Takeuchi Y, Nakayama K, Matsumoto T. Differentiation and cell surface expression of transforming growth factor-beta receptors are regulated by interaction with matrix collagen in murine osteoblastic cells. *J Biol Chem.* (1996) 271 (7):3938–44. doi: 10.1074/jbc.271.7.3938

16. Mizuno M, Fujisawa R, Kuboki Y. Type I collagen-induced osteoblastic differentiation of bone-marrow cells mediated by collagen-alpha2beta1 integrin interaction. *J Cell Physiol.* (2000) 184(2):207–13. doi: 10.1002/1097-4652(200008)184:2<207::AID-JCP8>3.0.CO;2-U

17. Shekaran A, Shoemaker JT, Kavanaugh TE, Lin AS, LaPlaca MC, Fan Y, et al. The effect of conditional inactivation of beta 1 integrins using twist 2 cre, osterix cre and osteocalcin cre lines on skeletal phenotype. *Bone.* (2014) 68:131–41. doi: 10.1016/j.bone.2014.08.008

18. Brunner M, Mandier N, Gautier T, Chevalier G, Ribba AS, Guardiola P, et al. Beta1 integrins mediate the BMP2 dependent transcriptional control of osteoblast differentiation and osteogenesis. *PLoS One.* (2018) 13(4):e0196021. doi: 10.1371/journal.pone.0196021

19. Aszodi A, Hunziker EB, Brakebusch C, Fassler R. Beta1 integrins regulate chondrocyte rotation, G1 progression, and cytokinesis. *Genes Dev* (2003) 17 (19):2465–79. doi: 10.1101/gad.277003

20. Huhtala M, Heino J, Casciari D, de Luise A, Johnson MS. Integrin evolution: insights from ascidian and teleost fish genomes. *Matrix Biol.* (2005) 24(2):83–95. doi: 10.1016/j.matbio.2005.01.003

21. Heino J. The collagen family members as cell adhesion proteins. *Bioessays.* (2007) 29(10):1001–10. doi: 10.1002/bies.20636

22. Unsoeld T, Taylor J, Hutter H. Discoidin domain receptors in invertebrates. In: Fridman R, Huang P, editors. *Discoidin domain receptors in health and disease.* New York, N.Y: Springer (2016). p. 87–105.

23. Chen L, Kong X, Fang Y, Paunikar S, Wang X, Brown JAL, et al. Recent advances in the role of discoidin domain receptor tyrosine kinase 1 and discoidin domain receptor tyrosine kinase 2 in breast and ovarian cancer. *Front Cell Dev Biol.* (2021) 9:747314. doi: 10.3389/fcell.2021.747314

24. Majo S, Auguste P. The yin and yang of discoidin domain receptors (DDRs): implications in tumor growth and metastasis development. *Cancers.* (2021) 13(7): 1725–51. doi: 10.3390/cancers13071725

25. Leitinger B. Discoidin domain receptor functions in physiological and pathological conditions. *Int Rev Cell Mol Biol.* (2014) 310:39–87. doi: 10.1016/B978-0-12-800180-6.00002-5

26. Marquez J, Olaso E. Role of discoidin domain receptor 2 in wound healing. *Histol Histopathol.* (2014) 29(11):1355–64. doi: 10.14670/HH-29.1355

27. Shrivastava A, Radziejewski C, Campbell E, Kovac L, McGlynn M, Ryan TE, et al. An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptors. *Mol Cell.* (1997) 1(1):25–34. doi: 10.1016/S1097-2765(00)80004-0

28. Vogel W, Gish GD, Alves F, Pawson T. The discoidin domain receptor tyrosine kinases are activated by collagen. *Mol Cell.* (1997) 1(1):13–23. doi: 10.1016/S1097-2765(00)80003-9

29. Leitinger B, Kwan AP. The discoidin domain receptor DDR2 is a receptor for type X collagen. *Matrix Biol.* (2006) 25(6):355–64. doi: 10.1016/j.matbio.2006.05.006

30. Rammal H, Saby C, Magnien K, Van-Gulick L, Garnotel R, Buache E, et al. Discodin domain receptors: potential actors and targets in cancer. *Front Pharmacol.* (2016) 7:55. doi: 10.3389/fphar.2016.00055

31. Leitinger B. Molecular analysis of collagen binding by the human discoidin domain receptors, DDR1 and DDR2. Identification of collagen binding sites in DDR2. *J Biol Chem.* (2003) 278(19):16761–9. doi: 10.1074/jbc.M301370200

32. Leitinger B, Steplewski A, Fertala A. The D2 period of collagen II contains a specific binding site for the human discoidin domain receptor, DDR2. *J Mol Biol.* (2004) 344(4):993–1003. doi: 10.1016/j.jmb.2004.09.089

33. Konitsiotis AD, Raynal N, Bihan D, Hohenester E, Farndale RW, Leitinger B. Characterization of high affinity binding motifs for the discoidin domain receptor DDR2 in collagen. *J Biol Chem.* (2008) 283(11):6861–8. doi: 10.1074/jbc.M709290200

34. Xu H, Raynal N, Stathopoulos S, Myllyharju J, Farndale RW, Leitinger B. Collagen binding specificity of the discoidin domain receptors: binding sites on collagens II and III and molecular determinants for collagen IV recognition by DDR1. *Matrix Biol.* (2011) 30(1):16–26. doi: 10.1016/j.matbio.2010.10.004

35. Lisman T, Raynal N, Groeneveld D, Maddox B, Peachey AR, Huizinga EG, et al. A single high-affinity binding site for von willebrand factor in collagen III, identified using synthetic triple-helical peptides. *Blood.* (2006) 108(12):3753–6. doi: 10.1182/blood-2006-03-011965

36. Hohenester E, Sasaki T, Giudici C, Farndale RW, Bachinger HP. Structural basis of sequence-specific collagen recognition by SPARC. *Proc Natl Acad Sci U S A.* (2008) 105(47):18273–7. doi: 10.1073/pnas.0808452105

37. Knight CG, Morton LF, Peachey AR, Tuckwell DS, Farndale RW, Barnes MJ. The collagen-binding A-domains of integrins alpha(1)beta(1) and alpha(2)beta(1) recognize the same specific amino acid sequence, GFOGER, in native (triple-helical) collagens. *J Biol Chem.* (2000) 275(1):35–40. doi: 10.1074/jbc.275.1.35

38. Barczyk M, Carracedo S, Gullberg D. Integrins. *Cell Tissue Res.* (2010) 339 (1):269–80. doi: 10.1007/s00441-009-0834-6

39. Carafoli F, Bihan D, Stathopoulos S, Konitsiotis AD, Kvansakul M, Farndale RW, et al. Crystallographic insight into collagen recognition by discoidin domain receptor 2. *Structure.* (2009) 17(12):1573–81. doi: 10.1016/j.str.2009.10.012

40. Mihai C, Chotani M, Elton TS, Agarwal G. Mapping of DDR1 distribution and oligomerization on the cell surface by FRET microscopy. *J Mol Biol.* (2009) 385 (2):432–45. doi: 10.1016/j.jmb.2008.10.067

41. Noordeen NA, Carafoli F, Hohenester E, Horton MA, Leitinger B. A transmembrane leucine zipper is required for activation of the dimeric receptor tyrosine kinase DDR1. *J Biol Chem.* (2006) 281(32):22744–51. doi: 10.1074/jbc.M603233200

42. Agarwal G, Smith AW, Jones B. Discoidin domain receptors: micro insights into macro assemblies. *Biochim Biophys Acta Mol Cell Res.* (2019) 1866(11):118496. doi: 10.1016/j.bbamcr.2019.06.010

43. Blissett AR, Garbellini D, Calomeni EP, Mihai C, Elton TS, Agarwal G. Regulation of collagen fibrillogenesis by cell-surface expression of kinase dead DDR2. *J Mol Biol*. (2009) 385(3):902–11. doi: 10.1016/j.jmb.2008.10.060

44. Flynn LA, Blissett AR, Calomeni EP, Agarwal G. Inhibition of collagen fibrillogenesis by cells expressing soluble extracellular domains of DDR1 and DDR2. *J Mol Biol*. (2010) 395(3):533–43. doi: 10.1016/j.jmb.2009.10.073

45. Alves F, Vogel W, Mossie K, Millauer B, Hofler H, Ullrich A. Distinct structural characteristics of discoidin I subfamily receptor tyrosine kinases and complementary expression in human cancer. *Oncogene*. (1995) 10(3):609–18. PMID: 7845687

46. Vogel WF, Aszodi A, Alves F, Pawson T. Discoidin domain receptor 1 tyrosine kinase has an essential role in mammary gland development. *Mol Cell Biol*. (2001) 21 (8):2906–17. doi: 10.1128/MCB.21.8.2906-2917.2001

47. Chou LY, Chen CH, Lin YH, Chuang SC, Chou HC, Lin SY, et al. Discoidin domain receptor 1 regulates endochondral ossification through terminal differentiation of chondrocytes. *FASEB J*. (2020) 34(4):5767–81. doi: 10.1096/fj.201901852RR

48. Chou LY, Chen CH, Chuang SC, Cheng TL, Lin YH, Chou HC, et al. Discoidin domain receptor 1 regulates Runx2 during osteogenesis of osteoblasts and promotes bone ossification via phosphorylation of p38. *Int J Mol Sci*. (2020) 21(19):7210–26. PMID: 33003599

49. Chou HC, Lin SY, Chou LY, Ho ML, Chuang SC, Cheng TL, et al. Ablation of discoidin domain receptor 1 provokes an osteopenic phenotype by regulating osteoblast/osteocyte autophagy and apoptosis. *Biomedicines*. (2022) 10(9):2173–87. doi: 10.3390/biomedicines10092173

50. Chavez MB, Kolli TN, Tan MH, Zachariadou C, Wang C, Embree MC, et al. Loss of discoidin domain receptor 1 predisposes mice to periodontal breakdown. *J Dent Res*. (2019) 98(13):1521–31. doi: 10.1177/0022034519881136

51. Labrador JP, Azcoitia V, Tuckermann J, Lin C, Olaso E, Manes S, et al. The collagen receptor DDR2 regulates proliferation and its elimination leads to dwarfism. *EMBO Rep*. (2001) 2(5):446–52. doi: 10.1093/embo-reports/kve094

52. Mohamed FF, Ge C, Cowling RT, Lucas D, Hallett SA, Ono N, et al. The collagen receptor, discoidin domain receptor 2, functions in Gli1-positive skeletal progenitors and chondrocytes to control bone development. *Bone Res*. (2022) 10 (1):11. doi: 10.1038/s41413-021-00182-w

53. Mohamed FF, Ge C, Hallett SA, Bancroft AC, Cowling RT, Ono N, et al. Control of craniofacial development by the collagen receptor, discoidin domain receptor 2. *Elife*. (2023) 12:e77257. doi: 10.7554/eLife.77257

54. Yang H, Sun L, Cai W, Gu J, Xu D, Deb A, et al. DDR2, A discoidin domain receptor, is a marker of periosteal osteoblast and osteoblast progenitors. *J Bone Miner Metab*. (2020) 38:670–7. doi: 10.1007/s00774-020-01108-y

55. Zeltz C, Gullberg D. The integrin-collagen connection–a glue for tissue repair? *J Cell Sci*. (2016) 129(4):653–64. doi: 10.1242/jcs.180992

56. Camper L, Holmvall K, Wangnerud C, Aszodi A, Lundgren-Akerlund E. Distribution of the collagen-binding integrin alpha10beta1 during mouse development. *Cell Tissue Res*. (2001) 306(1):107–16. doi: 10.1007/s004410100385

57. Mohamed FF, Ge C, Binrayes A, Franceschi RT. The role of discoidin domain receptor 2 in tooth development. *J Dent Res*. (2020) 99(2):214–22. doi: 10.1177/0022034519892563

58. Ge C, Mohamed F, Binrayes A, Kapila S, Franceschi RT. Selective role of discoidin domain receptor 2 in murine temporomandibular joint development and aging. *J Dent Res*. (2018) 97(3):321–8. doi: 10.1177/0022034517738190

59. Shi Y, He G, Lee WC, McKenzie JA, Silva MJ, Long F. Gli1 identifies osteogenic progenitors for bone formation and fracture repair. *Nat Commun*. (2017) 8(1):2043. doi: 10.1038/s41467-017-02171-2

60. Zhao H, Feng J, Ho TV, Grimes W, Urata M, Chai Y. The suture provides a niche for mesenchymal stem cells of craniofacial bones. *Nat Cell Biol*. (2015) 17 (4):386–96. doi: 10.1038/ncb3139

61. Greenblatt M, Bok S, Yallowitz A, McCormick J, Cung M, Sun J, et al. A multi-stem cell basis for craniosynostosis and calvarial mineralization. *Res Sq*. (2021). doi: 10.21203/rs.3.rs-1061838/v1

62. Debnath S, Yallowitz AR, McCormick J, Lalani S, Zhang T, Xu R, et al. Discovery of a periosteal stem cell mediating intramembranous bone formation. *Nature*. (2018) 562(7725):133–9. doi: 10.1038/s41586-018-0554-8

63. Lin KL, Chou CH, Hsieh SC, Hwa SY, Lee MT, Wang FF. Transcriptional upregulation of DDR2 by ATF4 facilitates osteoblastic differentiation through p38 MAPK-mediated Runx2 activation. *J Bone Miner Res*. (2010) 25(11):2489–503. doi: 10.1002/jbmr.159

64. Zhang Y, Su J, Yu J, Bu X, Ren T, Liu X, et al. An essential role of discoidin domain receptor 2 (DDR2) in osteoblast differentiation and chondrocyte maturation via modulation of Runx2 activation. *J Bone Miner Res*. (2011) 26 (3):604–17. doi: 10.1002/jbmr.225

65. Ge C, Wang Z, Zhao G, et al. Discoidin receptor 2 controls bone formation and marrow adipogenesis. *J Bone Miner Res*. (2016) 31(12):2193–203. doi: 10.1002/jbmr.2893

66. Borochowitz Z, Langer LO Jr, Gruber HE, Lachman R, Katznelson MB, Rimoin DL. Spondylo-meta-epiphyseal dysplasia (SMED), short limb-hand type: a congenital familial skeletal dysplasia with distinctive features and histopathology. *Am J Med Genet*. (1993) 45(3):320–6. doi: 10.1002/ajmg.1320450308

67. Bargal R, Cormier-Daire V, Ben-Neriah Z, Le Merrer M, Sosna J, Melki J, et al. Mutations in DDR2 gene cause SMED with short limbs and abnormal calcifications. *Am J Hum Genet*. (2009) 84(1):80–4. doi: 10.1016/j.ajhg.2008.12.004

68. Al-Kindi A, Kizhakkedath P, Xu H, John A, Sayegh AA, Ganesh A, et al. A novel mutation in DDR2 causing spondylo-meta-epiphyseal dysplasia with short limbs and abnormal calcifications (SMED-SL) results in defective intra-cellular trafficking. *BMC Med Genet*. (2014) 15:42. doi: 10.1186/1471-2350-15-42

69. Urel-Demir G, Simsek-Kiper PO, Akgun-Dogan O, Gocmen R, Wang Z, Matsumoto N, et al. Further expansion of the mutational spectrum of spondylo-meta-epiphyseal dysplasia with abnormal calcification. *J Hum Genet*. (2018) 63 (9):1003–7. doi: 10.1038/s10038-018-0473-4

70. Ali BR, Xu H, Akawi NA, John A, Karuvantevida NS, Langer R, et al. Trafficking defects and loss of ligand binding are the underlying causes of all reported DDR2 missense mutations found in SMED-SL patients. *Hum Mol Genet*. (2010) 19 (11):2239–50. doi: 10.1093/hmg/ddq103

71. Langer LO Jr, Wolfson BJ, Scott CI Jr, Reid CS, Schidlow DV, Millar EA, et al. Further delineation of spondylo-meta-epiphyseal dysplasia, short limb-abnormal calcification type, with emphasis on diagnostic features. *Am J Med Genet*. (1993) 45 (4):488–500. doi: 10.1002/ajmg.1320450419

72. Dias C, Cairns R, Patel MS. Sudden death in spondylo-meta-epiphyseal dysplasia, short limb-abnormal calcification type. *Clin Dysmorphol*. (2009) 18 (1):25–9. doi: 10.1097/MCD.0b013e3283186907

73. Xu L, Jensen H, Johnston JJ, Di Maria E, Kloth K, Cristea I, et al. Recurrent, activating variants in the receptor tyrosine kinase DDR2 cause warburg-cinotti syndrome. *Am J Hum Genet*. (2018) 103(6):976–83. doi: 10.1016/j.ajhg.2018.10.013

74. Guo Y, Yang TL, Dong SS, Yan H, Hao RH, Chen XF, et al. Genetic analysis identifies DDR2 as a novel gene affecting bone mineral density and osteoporotic fractures in Chinese population. *PLoS One*. (2015) 10(2):e0117102. doi: 10.1371/journal.pone.0117102

75. Kano K, Marin de Evsikova C, Young J, Wnek C, Maddatu TP, Nishina PM, et al. A novel dwarfism with gonadal dysfunction due to loss-of-function allele of the collagen receptor gene, Ddr2, in the mouse. *Mol Endocrinol*. (2008) 22 (8):1866–80. doi: 10.1210/me.2007-0310

76. Dullin C, Missbach-Guentner J, Vogel WF, Grabbe E, Alves F. Semi-automatic classification of skeletal morphology in genetically altered mice using flat-panel volume computed tomography. *PLoS Genet*. (2007) 3(7):e118. doi: 10.1371/journal.pgen.0030118

77. Kreiborg S, Marsh JL, Cohen MM Jr, Liversage M, Pedersen H, Skovby F, et al. Comparative three-dimensional analysis of CT-scans of the calvaria and cranial base in apert and crouzon syndromes. *J Craniomaxillofac Surg*. (1993) 21(5):181–8. doi: 10.1016/S1010-5182(05)80478-0

78. Zhang Y, Su J, Wu S, Teng Y, Yin Z, Guo Y, et al. DDR2 (discoidin domain receptor 2) suppresses osteoclastogenesis and is a potential therapeutic target in osteoporosis. *Sci Signal*. (2015) 8(369):ra31. doi: 10.1126/scisignal.2005835

79. Liu Q, Wang X, Chen Y, Ma X, Kang X, He F, et al. Ablation of myeloid discoidin domain receptor 2 exacerbates arthritis and high fat diet induced inflammation. *Biochem Biophys Res Commun*. (2023) 649:47–54. doi: 10.1016/j.bbrc.2023.01.074

80. Dallas SL, Xie Y, Shiflett LA, Ueki Y. Mouse cre models for the study of bone diseases. *Curr Osteoporos Rep*. (2018) 16(4):466–77. doi: 10.1007/s11914-018-0455-7

81. Komori T. Runx2, an inducer of osteoblast and chondrocyte differentiation. *Histochem Cell Biol*. (2018) 149(4):313–23. doi: 10.1007/s00418-018-1640-6

82. Franceschi RT, Ge C. Control of the osteoblast lineage by mitogen-activated protein kinase signaling. *Curr Mol Biol Rep*. (2017) 3:122–32. doi: 10.1007/s40610-017-0059-5

83. Ge C, Xiao G, Jiang D, Franceschi RT. Critical role of the extracellular signal-regulated kinase-MAPK pathway in osteoblast differentiation and skeletal development. *J Cell Biol*. (2007) 176(5):709–18. doi: 10.1083/jcb.200610046

84. Greenblatt MB, Shim JH, Zou W, Sitara D, Schweitzer M, Hu D, et al. The p38 MAPK pathway is essential for skeletogenesis and bone homeostasis in mice. *J Clin Invest*. (2010) 120(7):2457–73. doi: 10.1172/JCI42285

85. Li Y, Ge C, Franceschi RT. MAP kinase-dependent RUNX2 phosphorylation is necessary for epigenetic modification of chromatin during osteoblast differentiation. *J Cell Physiol*. (2017) 232(9):2427–35. doi: 10.1002/jcp.25517

86. Ge C, Xiao G, Jiang D, Yang Q, Hatch NE, Roca H, et al. Identification and functional characterization of ERK/MAPK phosphorylation sites in the Runx2 transcription factor. *J Biol Chem*. (2009) 284(47):32533–43. doi: 10.1074/jbc.M109.040980

87. Ge C, Zhao G, Li B, Li Y, Cawthorn WP, MacDougald OA, et al. Genetic inhibition of PPARgamma S112 phosphorylation reduces bone formation and stimulates marrow adipogenesis. *Bone*. (2018) 107:1–9. doi: 10.1016/j.bone.2017.10.023

88. Binrayes A, Ge C, Mohamed F, Kozloff KM, Franceschi RT. Role of discoidin domain receptor 2 in bone regeneration. *J Bone MinerRes*. (2018) 33(Abstract):S0448.

89. Binrayes A, Ge C, Mohamed FF, Franceschi RT. Role of discoidin domain receptor 2 in craniofacial bone regeneration. *J Dent Res*. (2021) 100(12):1359–66. doi: 10.1177/00220345211007447

90. Cooke ME, Hussein AI, Lybrand KE, Wulff A, Simmons E, Choi JH, et al. Correlation between RUST assessments of fracture healing to structural and biomechanical properties. *J Orthop Res*. (2018) 36(3):945–53. doi: 10.1002/jor.23710

91. Donnelly E, Ascenzi MG, Farnum C. Primary cilia are highly oriented with respect to collagen direction and long axis of extensor tendon. *J Orthop Res*. (2010) 28(1):77–82. doi: 10.1002/jor.20946

92. Bayer SV, Grither WR, Brenot A, Hwang PY, Barcus CE, Ernst M, et al. DDR2 controls breast tumor stiffness and metastasis by regulating integrin mediated mechanotransduction in CAFs. *Elife*. (2019) 8:e45508–32. doi: 10.7554/eLife.45508

93. Pagani CA, Bancroft AC, Tower RJ, Livingston N, Sun Y, Hong JY, et al. Discoidin domain receptor 2 regulates aberrant mesenchymal lineage cell fate and matrix organization. *Sci Adv*. (2022) 8(51):eabq6152. doi: 10.1126/sciadv.abq6152

94. Block GA. Control of serum phosphorus: implications for coronary artery calcification and calcific uremic arteriolopathy (calciphylaxis). *Curr Opin Nephrol Hypertens*. (2001) 10(6):741–7. doi: 10.1097/00041552-200111000-00003

95. Speer MY, Yang HY, Brabb T, Leaf E, Look A, Lin WL, et al. Smooth muscle cells give rise to osteochondrogenic precursors and chondrocytes in calcifying arteries. *Circ Res*. (2009) 104(6):733–41. doi: 10.1161/CIRCRESAHA.108.183053

96. Sun Y, Byon CH, Yuan K, Chen J, Mao X, Heath JM, et al. Smooth muscle cell-specific Runx2 deficiency inhibits vascular calcification. *Circ Res*. (2012) 111 (5):543–52. doi: 10.1161/CIRCRESAHA.112.267237

97. Ahmad PJ, Trcka D, Xue S, Franco C, Speer MY, Giachelli CM, et al. Discoidin domain receptor-1 deficiency attenuates atherosclerotic calcification and smooth muscle cell-mediated mineralization. *Am J Pathol*. (2009) 175(6):2686–96. doi: 10.2353/ajpath.2009.080734

98. Lino M, Wan MH, Rocca AS, Ngai D, Shobeiri N, Hou G, et al. Diabetic vascular calcification mediated by the collagen receptor discoidin domain receptor 1 via the phosphoinositide 3-kinase/akt/runt-related transcription factor 2 signaling axis. *Arterioscler Thromb Vasc Biol*. (2018) 38(8):1878–89. doi: 10.1161/ATVBAHA.118.311238

99. Ngai D, Mohabeer AL, Mao A, Lino M, Bendeck MP. Stiffness-responsive feedback autoregulation of DDR1 expression is mediated by a DDR1-YAP/TAZ axis. *Matrix Biol*. (2022) 110:129–40. doi: 10.1016/j.matbio.2022.05.004

100. Chen Y, Zhao X, Wu H. Arterial stiffness: a focus on vascular calcification and its link to bone mineralization. *Arterioscler Thromb Vasc Biol*. (2020) 40(5):1078–93. doi: 10.1161/ATVBAHA.120.313131

101. Arden N, Nevitt MC. Osteoarthritis: epidemiology. *Best Pract Res Clin Rheumatol*. (2006) 20(1):3–25. doi: 10.1016/j.berh.2005.09.007

102. Liu F, Steinkeler A. Epidemiology, diagnosis, and treatment of temporomandibular disorders. *Dent Clin North Am*. (2013) 57(3):465–79. doi: 10.1016/j.cden.2013.04.006

103. Wiberg B, Wanman A. Signs of osteoarthrosis of the temporomandibular joints in young patients: a clinical and radiographic study. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. (1998) 86(2):158–64. doi: 10.1016/S1079-2104(98)90118-4

104. Benjamin M, Ralphs JR. Biology of fibrocartilage cells. *Int Rev Cytol*. (2004) 233:1–45. doi: 10.1016/S0074-7696(04)33001-9

105. Schminke B, Muhammad H, Bode C, Sadowski B, Gerter R, Gersdorff N, et al. A discoidin domain receptor 1 knock-out mouse as a novel model for osteoarthritis of the temporomandibular joint. *Cell Mol Life Sci*. (2014) 71(6):1081–96. doi: 10.1007/s00018-013-1436-8

106. Xu L, Polur I, Servais JM, Hsieh S, Lee PL, Goldring MB, et al. Intact pericellular matrix of articular cartilage is required for unactivated discoidin domain receptor 2 in the mouse model. *Am J Pathol*. (2011) 179(3):1338–46. doi: 10.1016/j.ajpath.2011.05.023

107. Xu L, Servais J, Polur I, Kim D, Lee PL, Chung K, et al. Attenuation of osteoarthritis progression by reduction of discoidin domain receptor 2 in mice. *Arthritis Rheum*. (2010) 62(9):2736–44. doi: 10.1002/art.27582

108. Manning LB, Li Y, Chickmagalur NS, Li X, Xu L. Discoidin domain receptor 2 as a potential therapeutic target for development of disease-modifying osteoarthritis drugs. *Am J Pathol*. (2016) 186(11):3000–10. doi: 10.1016/j.ajpath.2016.06.023

109. Dey D, Wheatley BM, Cholok D, Agarwal S, Yu PB, Levi B, et al. The traumatic bone: trauma-induced heterotopic ossification. *Transl Res*. (2017) 186:95–111. doi: 10.1016/j.trsl.2017.06.004

110. Cawthorn WP, Scheller EL, Learman BS, Parlee SD, Simon BR, Mori H, et al. Bone marrow adipose tissue is an endocrine organ that contributes to increased circulating adiponectin during caloric restriction. *Cell Metab*. (2014) 20(2):368–75. doi: 10.1016/j.cmet.2014.06.003

111. Li Z, Bowers E, Zhu J, Yu H, Hardij J, Bagchi DP, et al. Lipolysis of bone marrow adipocytes is required to fuel bone and the marrow niche during energy deficits. *Elife*. (2022) 11:e78496. doi: 10.7554/eLife.78496

112. Zurakowski H, Gagnon A, Landry A, Layne MD, Sorisky A. Discoidin domain receptor 2 impairs insulin-stimulated insulin receptor substrate-1 tyrosine phosphorylation and glucose uptake in 3T3-L1 adipocytes. *Horm Metab Res*. (2007) 39(8):575–81. doi: 10.1055/s-2007-985132

113. Yang X, Li J, Zhao L, Chen Y, Cui Z, Xu T, et al. Targeting adipocytic discoidin domain receptor 2 impedes fat gain while increasing bone mass. *Cell Death Differ*. (2022) 29(4):737–49. doi: 10.1038/s41418-021-00887-9

114. Wang Z, Zhang Y, Pinkas DM, Fox AE, Luo J, Huang H, et al. Design, synthesis, and biological evaluation of 3-(imidazo[1,2- a]pyrazin-3-ylethynyl)-4-isopropyl- N-(3-((4-methylpiperazin-1-yl)methyl)-5-(trifluoromethyl)phenyl) benzamide as a dual inhibitor of discoidin domain receptors 1 and 2. *J Med Chem*. (2018) 61(17):7977–90. doi: 10.1021/acs.jmedchem.8b01045

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 02 August 2023
DOI 10.3389/fendo.2023.1136117



OPEN ACCESS

EDITED BY
Bjorn Olsen,
Harvard Medical School, United States

REVIEWED BY
Henrique Ballassini Abdalla,
São Leopoldo Mandic School, Brazil
Thomas G. H. Diekwisch,
University of Rochester Medical Center, United States

*CORRESPONDENCE
Dana T. Graves
dtgraves@upenn.edu

RECEIVED 02 January 2023
ACCEPTED 12 June 2023
PUBLISHED 02 August 2023

CITATION
Alharbi MA and Graves DT (2023) FOXO 1 deletion in chondrocytes rescues diabetes-impaired fracture healing by restoring angiogenesis and reducing apoptosis. *Front. Endocrinol.* 14:1136117. doi: 10.3389/fendo.2023.1136117



# FOXO 1 deletion in chondrocytes rescues diabetes-impaired fracture healing by restoring angiogenesis and reducing apoptosis

Mohammed A. Alharbi[1,2] and Dana T. Graves[2]*

[1]Department of Endodontics, Faculty of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia, [2]Department of Periodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, United States

**Introduction:** Diabetes mellitus is associated with higher risks of long bone and jaw fractures. It is also associated with a higher incidence of delayed union or non-union. Our previous investigations concluded that a dominant mechanism was the premature loss of cartilage during endochondral bone formation associated with increased osteoclastic activities. We tested the hypothesis that FOXO1 plays a key role in diabetes-impaired angiogenesis and chondrocyte apoptosis.

**Methods:** Closed fractures of the femur were induced in mice with lineage-specific FOXO1 deletion in chondrocytes. The control group consisted of mice with the FOXO1 gene present. Mice in the diabetic group were rendered diabetic by multiple streptozotocin injections, while mice in the normoglycemic group received vehicle. Specimens were collected 16 days post fracture. The samples were fixed, decalcified, and embedded in paraffin blocks for immunostaining utilizing anti cleaved caspase-3 or CD31 specific antibodies compared with matched control IgG antibody, and apoptosis by the TUNEL assay. Additionally, ATDC5 chondrocytes were examined in vitro by RT-PCR, luciferase reporter and chromatin immunoprecipitation assays.

**Results:** Diabetic mice had ~ 50% fewer blood vessels compared to normoglycemic mice FOXO1 deletion in diabetic mice partially rescued the low number of blood vessels ($p < 0.05$). Additionally, diabetes increased caspase-3 positive and apoptotic chondrocytes by 50%. FOXO1 deletion in diabetic animals blocked the increase in both to levels comparable to normoglycemic animals ($p < 0.05$). High glucose (HG) and high advanced glycation end products (AGE) levels stimulated FOXO1 association with the caspase-3 promoter in vitro, and overexpression of FOXO1 increased caspase-3 promoter activity in luciferase reporter assays. Furthermore, we review previous mechanistic studies demonstrating that tumor necrosis factor (TNF)

**Abbreviations:** AKT, Ak strain transformin; BSA, Bovine Serum Albumin; DMEM, Dulbecco's modified Eagle's medium; EDTA, Ethylenediaminetetraacetic acid; ECM, Extracellular matrix; FOXO1, Forkhead box 01; IL-, Interleukin; Prg, Proteoglycan; RANKL, Receptor Activator of NFκB Ligand; STZ, Streptozocin; TNF-α, Tumor Necrosis Factor; VEGFA, Vascular Endothelial Growth Factor A.

inhibition reverses impaired angiogenesis and reverses high levels of chondrocyte apoptosis that occur in fracture healing.

**Discussion:** New results presented here, in combination with recent studies, provide a comprehensive overview of how diabetes, through high glucose levels, AGEs, and increased inflammation, impair the healing process by interfering with angiogenesis and stimulating chondrocyte apoptosis. FOXO1 in diabetic fractures plays a negative role by reducing new blood vessel formation and increasing chondrocyte cell death which is distinct from its role in normal fracture healing.

KEYWORDS

animal model, bone, inflammation, neovascularization, FOXO, T1DM, cartilage, apoptosis

# 1 Introduction

Fracture healing is a complex process that requires well-orchestrated and coordinated events that involve various cell types (1). An early and critical step in healing is the proliferation and recruitment of mesenchymal stem cells and their differentiation to chondrocytes, osteoblasts, and other cell types. Chondrocytes lay down cartilage to support and stabilize the fracture site (2). Our lab has recently shown that chondrocytes modulate osteoclastogenesis by producing receptor activator of nuclear factor kappa-β ligand (RANKL), which stimulates the removal of cartilage during the early stages of endochondral bone formation (3). This process coincides with neovascularization, which is essential for healing to progress and is negatively modulated by diabetes (4, 5). Consistent with this observation is significantly improved fracture healing with vascular endothelial growth factor (VEGF) stimulated angiogenesis (6). The formation of new blood vessels during fracture healing provides essential nutrition to the callus, helps recruit osteoclast precursors to resorb the cartilage and endothelial cells produce mediators to modulate the healing process (7). Cartilage resorption and chondrocyte apoptosis are critical steps in the transition from a cartilaginous callus to a hard bony callus needed for fracture healing (2, 3, 8).

Diabetes significantly interferes with the fracture repair process (9). Type 1 diabetes mellitus (T1DM) and T2DM are serious concerns worldwide as the incidence of both chronic diseases has increased in the past 20 years. Today it is estimated that 415 million people worldwide are diagnosed with diabetes (10). Diabetes is characterized by high blood glucose levels (hyperglycemia) due to insulin deficiency or due to the inability to respond to insulin. Several animal and human studies demonstrate that T1DM has an impact on bone with a 2-fold increase in fractures compared to non-diabetics (11), and T2DM patients have a 5-fold greater risk of vertebral fracture (12). Moreover, T1DM and T2DM impair the fracture healing process in humans and in diabetic animals (11, 13–15) and increases the risk of bone fracture (16–18). Furthermore, it delays, impairs, and increases the incidents of non-unions in both animals and humans (13, 19). Diabetes delays fracture healing in the jaw bones and is associated with increased mandibular fracture healing complications and increased recurrence of fractures (20, 21).

FOXO1 is a forkhead transcription factor box O family member, which regulates various cellular events, including proliferation, differentiation, apoptosis, and the response to oxidative stress (22). FOXO1, compared to the three other FOXO family members is more highly expressed in cartilage and bone and has a more dramatic impact on these tissues when deleted (23). FOXO1 has several important functions in chondrocytes. It has been recently shown that FOXO1 regulates chondrocyte homeostasis in a FOXO1 loss of function mouse (Agc1- CreERT2;FoxO1f/f) model. These mice exhibit histologic changes collectively indicative of an increased catabolic state (24). FOXO1 also maintains articular chondrocyte homeostasis through the induction of anabolic and autophagy-related gene expression. FOXO1 has a paramount role in protecting chondrocytes against oxidative stress via the ALK5–SMAD3 pathway (25). Additionally, FOXO1 regulates the expression of interleukin 6 (IL-6) in chondrocytes, which is a potent inflammatory mediator regulating (26). Animals with FOXO1 knockout in chondrocytes initially had more cartilage produced and later had greater loss of cartilage. The latter was associated with increased IL-6. These findings suggest that FOXO1 limits the early expansion of cartilage and prevents its loss at a later stage. Additionally, FOXO1 regulates proteoglycan4(Prg4) expression which is crucial fo rmaintaining cartilage integrity (27). Prg4KO mice showed a significan tincrease in chondrocytes apoptosis and cell loss (28, 29).

In the oral cavity, FOXO1 has been reported to play a pivotal role in temporomandibular joint (TMJ) osteoarthritis. When FOXO1 is inhibited by protein kinase B also known as Ak strain transformin (Akt), there is greater extracellular matrix (ECM) degradation as well as increased chondrocyte apoptosis in a TMJ osteoarthritis model (30). This findings suggests that Foxo1 plays a protective role in TMJ osteoarthritis.

We have shown that FOXO1 plays a positive role in promoting chondrocyte function to facilitate normal fracture healing. When

FOXO1 is deleted in chondrocytes (Col2a1.Cre$^{+}$.FOXO1$^{L/L}$) in normal conditions, there is a reduction in blood vessel formation and a reduction in the capacity of chondrocytes to induce microvascular endothelial cell tube formation *in vitro* (31). This can be mechanistically explained by the significant reduction in the VEGFA expression by chondrocytes upon FOXO1 deletion in these cells. The results are further supported by evidence that FOXO1 binds to the VEGFA promoter in chondrocytes and FOXO1 induces VEGFA transcriptional activity (31).

*In vivo* results point to the importance of FOXO1 activity in chondrocytes in stimulating normal fracture healing and ultimately endochondral bone formation in adult animals. However, in diabetic fracture healing, FOXO1 plays a negative role in the healing process (3, 32). The goal of this report is to identify mechanisms by which FOXO1 can have a negative role in fracture repair through its detrimental impact on chondrocyte function.

# 2 Materials and methods

## 2.1 Animals and diabetes induction

All animal studies were carried out with approval from the University of Pennsylvania.

Institutional Animal Care and Use Committee (IACUC) (Protocol # 803894) and the Guide for the care and use of laboratory animals, eighth edition (2011), were followed. FOXOL/L mice were provided by R.A. DePinho (MD Anderson Cancer Center, Houston, TX) and created as previously described (33). The experimental group included mice with lineage-specific FOXO1 deletion in chondrocytes (Col2α1Cre$^{+}$.FOXO1$^{L/L}$) and results were compared with Col2α1Cre$^{-}$.FOXO1$^{L/L}$ littermate controls. All animals were monitored daily by University Laboratory Animal Services (ULAR) and cages were changed weekly with 5001 Rodent Diet. (Purina Lab Diet, St. Louis, MO). Every cage contained two to five mice under standard conditions with 14-hours light/10-hours dark cycles. Prior to starting the experiment, genotyping was performed via PCR using both Cre and FOXO1 primers using genomic DNA extracted from the mice's tails/ears. The results were also verified at the end of the experiment. Type-1 diabetes was induced as described by us (34) and developed originally by Like and Rossini (35). Intraperitoneal (IP) streptozotocin (STZ) injections (40 mg/kg; Sigma-Aldrich, St. Louis, Missouri, US) in 10mM citrate buffer were given once every day for five consecutive days. Vehicle alone was used for control mice. Ten days after the last injection, blood glucose levels were measured weekly from small lacerations in the mice tails from both groups. Mice with two consecutive blood glucose readings of >220 mg/dl were considered diabetic. The mice had been diabetic for at least three weeks prior to starting the experiment.

## 2.2 Fractures induction and sample preparations

At 12-14 weeks old, a simple transverse fracture was induced. as previously described (36). Briefly, an incision was made in the knee and a 30-gauge spinal needle was inserted for fixation. A controlled, closed, simple, transverse fracture was created by blunt trauma to the middle of the femur at the mid-diaphyseal region. There was no change in the animal behavior noticed between the different groups. Animals were euthanized, and femurs were harvested 16 days after fracture. All the sites were evaluated radiographically and physically at euthanasia. Fractures that were not mid-diaphyseal or that were grossly comminuted were excluded from the experiment. Less than 5% of the samples were excluded. Fractured samples were fixed for 24 hours in cold 4% paraformaldehyde. Decalcification was achieved by incubating the samples at room temperature in 10% ethylenediaminetetraacetic acid (EDTA) solution for five weeks before embedding them in paraffin blocks. Transverse sections were prepared as described by us and initially by Gerstenfeld et al. (14, 36).

## 2.3 Immunofluoresence

After dewaxing and hydration, a pressure cooker (2100-Retriever Aptum, Southampton, UK) was used at 120°C for 20 minutes with 10mM of citric acid with a pH of 6.0 for antigen retrieval followed by non-specific binding blocking for 55 minutes, using nonimmune serum matching the secondary antibody. Slides were incubated overnight with CD-31 specific antibody (Abcam, ab28364), anti-cleaved caspase 3 antibody (Cell Signaling Technology 96615) or the appropriate isotype-matched negative control IgG (Vector, I-1000, Burlingame, CA). The primary antibody was localized by a biotinylated secondary antibody (Vector, BA-1000). To localize the antibody complex, Alexa Fluor 546–conjugated streptavidin (Invitrogen S-11225, Carlsbad, CA) was used. Nuclei were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO). Images were captured at different magnifications (40x, 200x, and 400x magnification) using a fluorescence microscope (ECLIPSE 90i; Nikon). The exposure time was set so that the IgG control had no signal. The quantification was performed with the aid of NIS Elements AR image analysis software. The unit of measure was the animal. Each value was calculated by examining six to eight animals in each group.

## 2.4 TUNEL assay

Apoptotic cells were detected by DeadEnd$^{TM}$ Colorimetric TUNEL System (Promega, WI, USA), which detects apoptotic cells by labeling and detecting DNA strand breaks by the TUNEL method. To distinguish apoptotic chondrocytes from other cells, the images were combined with a bright-field channel to confirm the cell morphology. In addition, the TUNEL-positive cells were compared with a safranin-o/fast green stain to verify the location of chondrocytes and define the entire region of interest. Slides were first deparaffinized and hydrated in the same manner mentioned. Slides were then incubated at room temperature for 15 minutes in diluted proteinase K solution and then rinsed with phosphate-buffered saline (PBS). This was followed by 5 minutes of incubation in endogenous oxidation-blocking solution, 3% hydrogen peroxide,

at room temperature and then rinsed in PBS. After that, slides were incubated at room temperature for 30 seconds in equilibration buffer, then in working strength TdT enzyme for an hour at 37°C. Slides were then incubated in working strength stop/wash buffer for 10 minutes at room temperature, and they were then rinsed in PBS and incubated in anti-digoxigenin conjugate for 30 minutes at 37°C. Slides were rinsed again in PBS and mounted with DAPI to stain the nuclei. The mean number of immunopositive cells was calculated for each group examining six to eight animals per group. The number of immunopositive cells was divided by the area or as a percentage of the total number of cells in the region of interest.

## 2.5 Cell culture, RNA extraction, qPCR

ATDC5 chondrocytes obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) were used for *in vitro* experiments. Cells were cultured in 50% Dulbecco's modified Eagle's medium (DMEM) (Gibco, Gaithersburg, MD, USA) and 50% F12 (Gibco) with 5% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic (Anti-Anti) (Thermo Fisher Scientific, Waltham, MA, US). Hypertrophic differentiation induction was performed using ascorbic acid (50mg/mL) for 6 days with increasing concentrations of NaH2PO4 0.5mM, 1mM, and 2mM (37). All cell cultures were maintained in a 5% CO2 humidified incubator at 37°C.

Quick-RNA MicroPrep kit (Zymo Research, Irvine, CA, USA) was used according to the manufacturer's instructions to isolate RNA. RNA was converted to cDNA using an ABI High- Capacity RNA-to-cDNA kit (Applied Biosystems; cat# 4387406). caspase3 mRNA levels were measured by qPCR in ATDC5 chondrocytes cultured in low glucose (5mM d-glucose) and high glucose (25mM d-glucose) for 5 days and transfected with FOXO1 siRNA or scrambled siRNA. qPCR was performed using ABI Fast SYBR Green Master Mix (cat# 4385612) and a StepOne Plus real-time PCR system (Applied Biosystems). Relative amounts were calculated using the ΔΔCt method. Data are expressed as percent input after quantitative amplification of equivalent amounts of DNA. Experiments were performed with triplicate replicates and carried out three times with similar results.

## 2.6 Dual-luciferase reporter

siRNA transfections with ATDC5s were performed at approximately 60-70% confluence in 6-well plates with 10nM siFOXO1 or scramble control (Dharmacon, Lafayette, CO) using GenMute transfection reagent (Rockville, MD) according to the manufacturer's instructions. Plasmid transfections were performed in OptiMEM (Gibco) medium with 1250ng plasmid in 3.75ul of Lipofectamine 3000 transfection reagent per well and following manufacturer's instructions with ATDC5s at approximately 60-70% confluence for 4.5 hours before being replaced with full media. To quantify caspase-3 expression, cells were co-transfected with treatment vectors: empty, ADA [Threonine 24 to Alanine (A) and Serine 253 to Aspartate (D) and Serine 316 to Alanine (A)], ADA + 6KQ (K242, K245, K259, K262, K271, and K291 were replaced with glutamine on ADA), ADA + 6KR (K242, K245, K259, K262, K271, and K291 were replaced with arginine on ADA), KQ(K242, K245, K259, K262, K271, and K291 were replaced with glutamine) or KR(K242, K245, K259, K262, K271, and K291 were replaced with arginine) mutants (Addgene, Cambridge, MA. pCMV5 backbone) along with a caspase-3 luciferase reporter (pGL4) as described and generously provided by Dr. Estrov (38). Co-transfection utilized the same transfection protocol with an expression vector, and the reporter was added at a 1:1 ratio of 150ng per well in a 48-well plate. Expression values were normalized using a Renilla control (pGL4) containing the CMV promoter at a 1:20 ratio. Results were quantified using a Dual-Luciferase Reporter Assay Kit from Promega (Madison, WI, cat# - E1960) and quantified on a Tecan Infinite M200. Experiments were performed with triplicate replicates and carried out three times with similar results.

## 2.7 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed with the ChIP-IT Kit (Active Motif, Carlsbad, CA) using approximately 1.5 x107 ATDC5 cells. The cells were cultured using multiple conditions, including 1) hypertrophic differentiation for 6 days using differentiation media as mentioned earlier; 2) cells at 70–80% treated with CML-BSA (200 mg/mL), an AGE, for 3 days or unmodified BSA (200 mg/mL) for a similar period; and 3) cells grown in High glucose (HG) (25 mmol/L) media for 5 days. Formaldehyde was used to fix the cells and nuclei obtained following Dounce homogenization. ChIP was performed following the manufacturer's instructions using an anti-FOXO1 antibody (5 mg) (SC-11350X; Santa Cruz Biotechnology) or control polyclonal non-specific IgG (Cell Signaling Technology). Protein G-coupled beads were used to purify the chromatin–antibody complexes. Three quantitative real-time PCR reactions for the caspase-3 promoter region, which contains FOXO1 consensus response elements, were done with similar results. Experiments were performed with triplicate replicates and carried out three times with similar results.

# 3 Statistics

All data were analyzed by one-way analysis of variance and differences between groups determined using Tukey's *post-hoc* tests unless otherwise stated. Student's t-test was used in some *in vitro* experiments where only two groups were compared. $P < 0.05$ was considered statistically significant. Data are expressed as the mean ± SEM.

# 4 Results

## 4.1 Diabetes and vascularization

We have previously examined the effect of diabetes on angiogenesis during fracture healing by identifying blood vessels with an antibody to Factor VIII or CD31 (39). As shown in Table 1, which was adapted from (39), there was a 65%–80% increase in the

TABLE 1 Diabetic-induced reduced angiogenesis is restored to normal levels upon insulin treatment and TNF α inhibition in diabetic fracture healing.

| A | | | |
|---|---|---|---|
| Factor V111 + small Vessels per mm2 | | | |
| Day 10 | Normoglycemic | Diabetic | Diabetic + insulin |
| | 518 | 265* | 447$^{+}$ |
| Day 16 | 896 | 497* | 764$^{+}$ |
| Factor V111 + Moderate Vessels per mm2 | | | |
| Day 10 | Normoglycemic | Diabetic | Diabetic + insulin |
| | 214 | 110* | 199$^{+}$ |
| Day 16 | 364 | 179* | 322$^{+}$ |
| B | | | |
| CD31 + small Vessels per mm2 | | | |
| Day 16 | Normoglycemic | Diabetic | Diabetic + PEG |
| | 107 | 58* | 94$^{+}$ |
| CD31 + Moderate Vessels per mm2 | | | |
| Day 16 | Normoglycemic | Diabetic | Diabetic + PEG |
| | 47 | 29* | 53$^{+}$ |

(1A) Factor VIII positive blood vessels were evaluated at Day 10 and 16 post-fracture by IHC in areas of new bone formation in normoglycemic, diabetic, and insulin-treated diabetic mice *Indicates a significant difference between normal and diabetic (P<0.05). + Indicates a significant difference between insulin-treated and untreated diabetic animals (P<0.05). (1B) CD31 immunopositive blood vessels were counted 16 days post fractures in areas of bone formation in normoglycemic, diabetic, and TNFα-specific inhibitor pegsunercept treated diabetic mice. *Indicates a significant difference between normal and diabetic (P<0.05). +Indicates a significant difference between PEG-treated and untreated diabetic animals (P<0.05). Original data found in ref (39).

number of blood vessels between day 10 and day 16 in normoglycemic mice ($p < 0.05$) (Table 1). Diabetes reduced the number of Factor VIII$^{+}$ small and moderate-sized blood vessels by almost half ($p > 0.05$) (Table 1). Mechanistically, this was related to the level of inflammation as shown by the rescue of diabetes-reduced neovascularization when a TNFα-specific inhibitor, pegsunercept (PEG), was applied.

Since we had shown that some of the effects of TNF could be directly related to the transcription factor FOXO1 (40), we examined the hypothesis that FOXO1 is a key factor in regulating angiogenesis in diabetic animals. This was accomplished by studying neovascularization in healing fractures in animals with chondrocyte-specific FOXO1 deletion in diabetic experimental (Col2a1Cre$^{+}$.FOXO1$^{L/L}$) compared to diabetic control (Col2a1Cre$^{-}$.FOXO1$^{L/L}$) littermates. Diabetes resulted in a ~ 50% reduction in the number of both small and moderately sized vessels compared to the WT (Figure 1, $P > 0.05$). Conditional FOXO1 deletion in chondrocytes in diabetic animals resulted in a partial and significant rescue of diabetes-inhibited formation of small and moderate-sized blood vessels (P<0.05).

## 4.2 Diabetes increases chondrocytes apoptosis via FOXO1-dependent mechanism

We have previously examined apoptotic cells in fracture healing using a TUNEL assay (40). Table 2, adapted from (40), demonstrated that the number of apoptotic chondrocytes was 5.4-fold higher in the diabetic group compared to the normoglycemic (P<0.05; Table 2). Insulin treatment significantly reduced most of this increase (P<0.05). When the entire callus was examined, the diabetic group showed a 2.5-fold increase in the total number of apoptotic cells compared to the normoglycemic, which was largely rescued by insulin treatment (P<0.05; Table 2). The increase in apoptosis was directly linked to the effect of TNF-α as demonstrated by a complete rescue when TNF-α was inhibited by pegsunercept. There was also no significant difference in the number of apoptotic chondrocytes between the diabetic and the normoglycemic group on day 10. Chondrocyte apoptosis increased further on day 16 post-fracture in the diabetic animals, and the increase was rescued to normal levels upon PEG treatment (P<0.05; Table 2).

We then examined whether the transcription of FOXO1 mediated apoptosis in diabetic fracture healing using the experimental animals described above. There was a ~ 50% increase in TUNEL-positive chondrocytes in the hypertrophic and the mixed zone that contains both cartilage and bone (Figures 2A–E, p<0.05). This increase was completely rescued to normal levels upon specific FOXO1 deletion in chondrocytes in diabetic animals (Figure 2F, p<0.05).

## 4.3 FOXO1 regulates caspase-3 under diabetic conditions

To further examine how diabetes could increase chondrocyte apoptosis, we measured the levels of cleaved caspase-3. *In vivo*

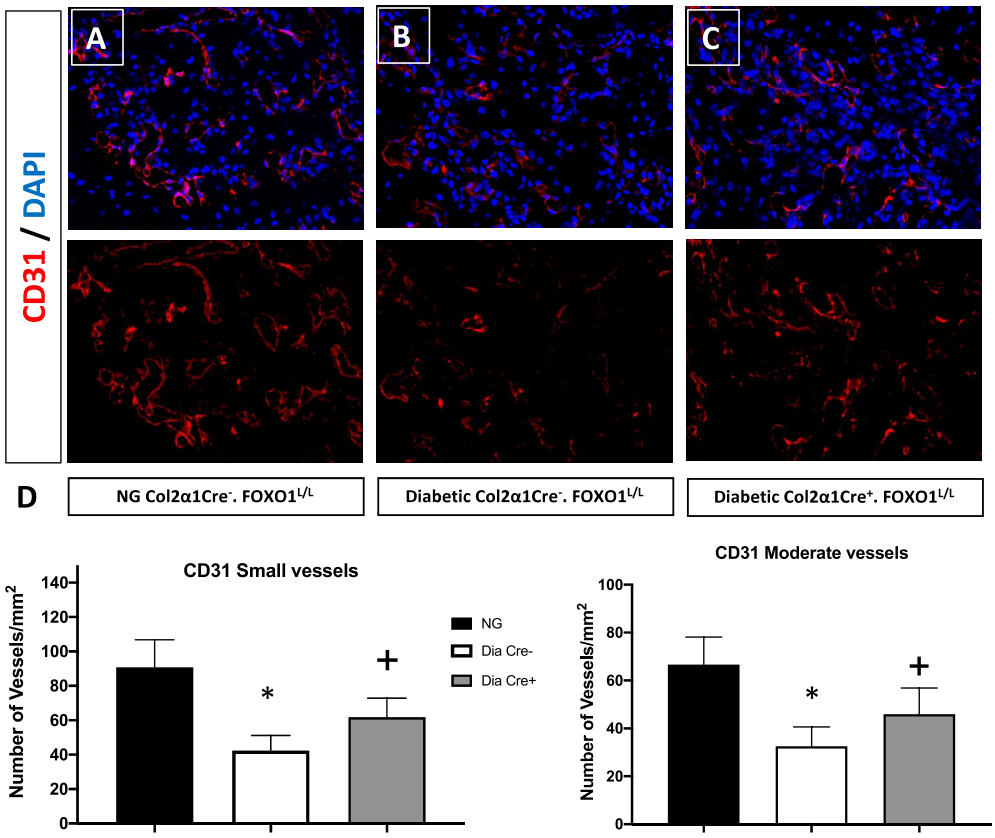



FIGURE 1
FOXO1 deletion in chondrocytes partially rescues diabetes impaired angiogenesis. Photmicrographs of histologic sections 16 days post fracture from **(A)** normoglycemic; **(B)** diabetic WT; and **(C)** diabetic FOXO1-deleted mice (Cre+.FOXO1LL). Immunofluorescence was carried out with CD31 specific-antibody to detect endothelial cells. No immunofluorescence was detected with control primary antibody (not shown). **(D)** Quantitative analysis of CD31 positive blood vessels in the transition zone. Blood vessels containing 2-4 CD31 positive cells were considered as small vessels while moderate-sized vessels contained 5-8 CD31 positive cells. Data are expressed as mean ± SEM. *indicates a significant difference between specimens from study group and matched normoglycemic animals. + indicates signficant difference between diabetic Cre-.FOXO1LL and diabetic Cre+.FOXO1LL groups. Significance was determined by ANOVA followed by Tukey's *post-hoc* test (P<0.05).

TABLE 2 Diabetes upregulated the number of apoptotic chondrocytes.

| A | | | |
|---|---|---|---|
| Apoptotic chondrocytes/mm2 of cartilage | Normoglycemic | Diabetic | Diabetic + insulin |
| | 0.5 | 2.7* | $1^{+}$ |
| Total Apoptotic cells/mm2 of callus | Normoglycemic | Diabetic | Diabetic + insulin |
| | 1.8 | 4.2* | $2^{+}$ |
| **B** | | | |
| Day 10 Apoptotic chondrocytes/mm2 of cartilage | Normoglycemic | Diabetic | |
| | 0.89 | 0.93* | |
| Day 16 Apoptotic chondrocytes/mm2 of cartilage | Normoglycemic | Diabetic | Diabetic + PEG |
| | 1.17 | 2.43* | $0.74^{+}$ |

(2A) Quantitative analysis of apoptotic cell numbers in normoglycemic, diabetic, and insulin-treated diabetic mice measured in TUNEL stained sections combined with safranin-O/fast green stain to distinguish chondrocytes. (2B) Sections from fracture calluses were examined by the TUNEL assay and counterstained with safranin-O/fast green to identify apoptotic chondrocytes in the cartilage area. Quantitative analysis of TUNEL-positive cells in diabetic mice, matched normoglycemic control mice, and diabetic mice were treated with pegsunercept (PEG) which was started 10 days after fracture. *Indicates a significant difference between normal and diabetic (P<0.05). + Indicates a significant difference between insulin-treated or pegsunercept-treated and untreated diabetic animals (P<0.05). Original data is found in ref (14).
Insulin treatment and TNFα inhibition rescued it to normal levels.

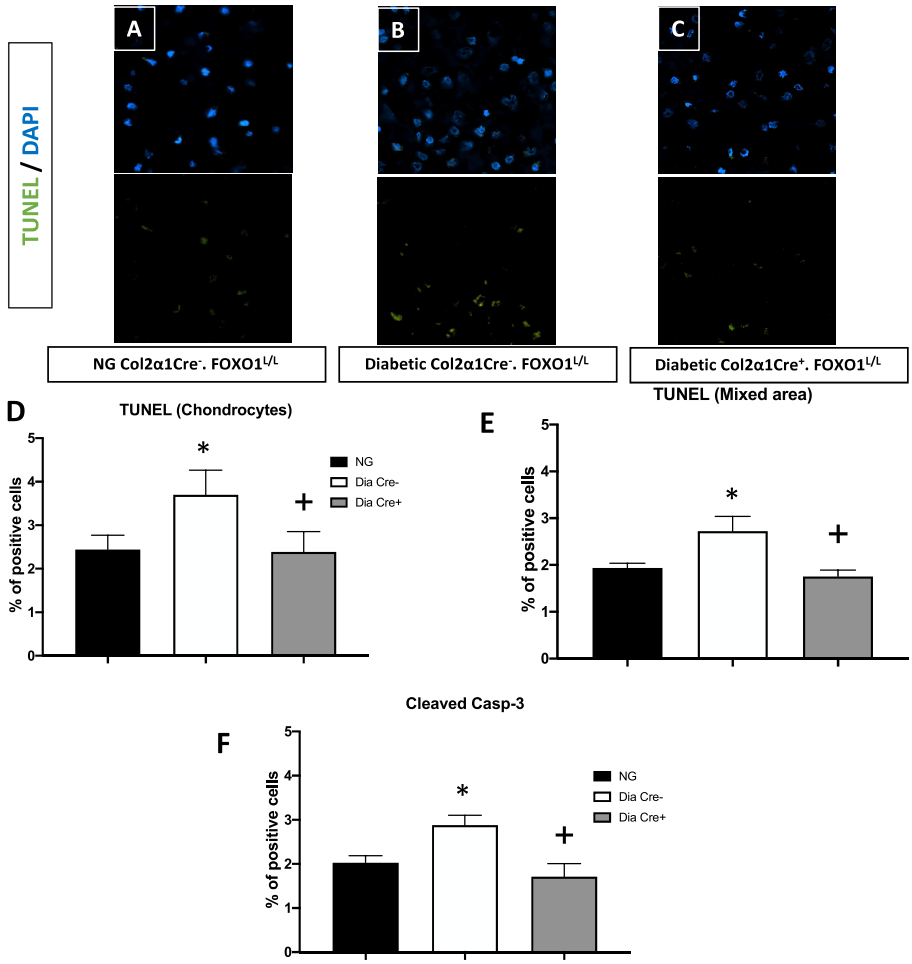



FIGURE 2
Diabetes significantly increases chondrocytes apoptosis, which is rescued by FOXO1 deletion in diabetic mice. Fracture sites were examined by a fluorescent TUNEL assay 16 days post fracture from **(A)** normoglycemic; **(B)** diabetic WT; and **(C)** diabetic FOXO1-deleted mice (Cre+.FOXO1LL). **(D, E)** Quantitative analysis of TUNEL positive cells in the cartilage area and transitional area containing both cartilage and bone, respectively. **(F)** Quantitative analysis of caspase-3 positive chondrocytes. Data are expressed as mean ± SEM. *indicates a significant difference between specimens from diabetic and matched normoglycemic animals; + indicates a significant difference between specimens from diabetic animals with FOXO1 deletion compared to littermate diabetic control animals. Significance was determined by ANOVA followed by Tukey's *post-hoc* test (P<0.05).

immunofluorescence with an antibody specific for cleaved caspase-3 showed that diabetes increased caspase-3 positive chondrocytes by ~50% compared to fracture healing in normoglycemic animals (Figure 2D, p<0.05). FOXO1 deletion in chondrocytes in diabetic animals reduced the over-expression of caspase-3 to levels similar to that of the normal group (Figure 2D, P>0.05).

We then directly assessed the role of FOXO1 in regulating caspase-3 expression by transfecting chondrocytes with FOXO1 siRNA or scrambled siRNA in low-glucose (LG) and high-glucose-containing media (HG). HG media resulted in a 2.7-fold increase in caspase-3 expression (Figure 3A, p<0.05). Silencing FOXO1 with siRNA significantly downregulated the increase in caspase-3 expression induced by HG. ChIP assays were undertaken to measure FOXO1 interaction with the caspase-3 promoter. HG stimulated an ~ 8-fold increase in FOXO1 binding to the caspase-3 promoter (Figure 3B, p<0.05). Treatment of chondrocytes with an advanced glycation end product (AGE), carboxymethyl lysine modified BSA, stimulated a similar increase. Luciferase reporter assays were undertaken to examine the direct effect of HG and AGE on caspase-3 promoter activity. Transfection of chondrocytes with a FOXO1 expression vector increased caspase-3 transcriptional activity 4-fold in the presence of HG compared to standard (LG) media and 6-fold in the presence of AGE compared to unmodified BSA (Figure 3C, p<0.05). Interestingly, the combination of HG or AGE with FOXO1 transfection was greater than the effect of FOXO1 transfection alone.

## 5 Discussion

Diabetic fracture healing is characterized by increased inflammation and oxidative stress, which reduces osteoblast proliferation and differentiation and interferes with the production of the osteoid matrix to limit the healing response (41). However, the role of chondrocytes in the healing has not been investigated as thoroughly. To better understand the mechanisms of how diabetes alters the

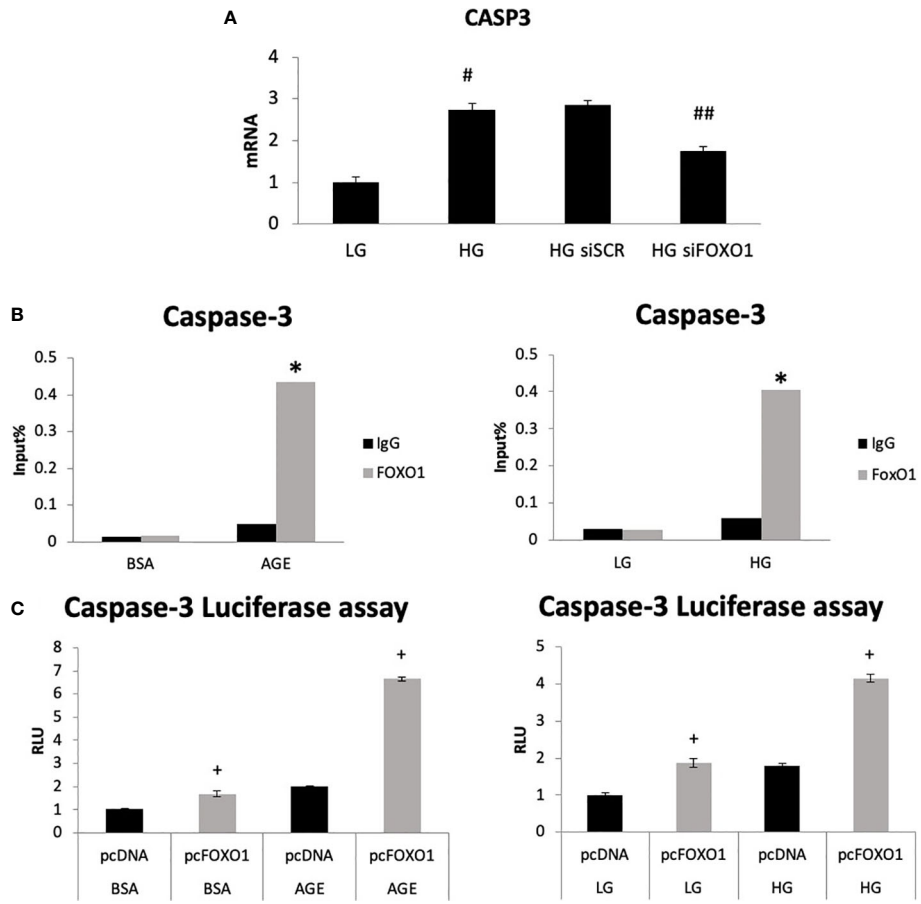



FIGURE 3
High glucose and an AGE modulate FOXO1 regulation of caspase-3. **(A)** Chondrocytes (ATDC5) were incubated in standard media (LG) or media supplemented with high glucose (HG) (25mM) for five days or 200ug/ml carboxymethylysine-modified BSA, an AGE or unmodified BSA for three days. Chromatin immunoprecipitation (ChIP) assays were performed by pull down with FOXO1 antibody or matched IgG. **(B)** Chondrocytes were co-transfected with a FOXO1 expressing plasmid and a caspase-3 luciferase reporter construct and incubated in standard media, HG media, and an AGE containing media. Luciferase activity was measured. **(C)** Chondrocytes were incubated in HG and transfected with siFOXO1 and or scrambled siRNA (siSCR). Caspase-3 mRNA level were analyzed by qPCR. Results are expressed as the mean ∓SE. *indicates $p < 0.05$ compared to control IgG group. $^{+}$indicates $p < 0.05$ compared to matched FOXO1 non-mutant. $^{\#}$Indicates $p < 0.05$ compared to LG group. $^{\#\#}$indicates $p < 0.05$ compared to siRNA control group.

healing, we examined the effect of diabetes on vascularization and apoptosis. We specifically looked at the regulatory role of the transcription factor FOXO1in chondrocytes in this process.

It is well known that diabetes impairs angiogenesis and wound healing (42). Our results indicate that diabetes significantly reduces the number of vessels in the fracture callus, as shown by the reduction in CD31-positive small and moderately-sized vessels studied in histologic samples (39). The impact of diabetes was directly related to the level of inflammation as inhibition of TNF rescued this effect. We add to this information by showing that FOXO1 also plays an important role in interfering with diabetic fracture healing. Reduced angiogenesis is clinically important since it can lead to delayed repair, as shown in an animal model with an ischemic fracture that impairs healing. (43). These results were unexpected since in normal healing, FOXO1 expression in chondrocytes leads to enhanced angiogenesis through the production of VEGFA (31). The downstream mechanisms that may account for the improvements in angiogenesis when FOXO1 is deleted in chondrocytes may be explained by a recent finding that FOXO1 deletion leads to increased inflammation in the fracture callus in diabetic animals (26). Thus, in normal fracture healing, FOXO1 may promote angiogenesis through the production of VEGFA, as shown in both soft tissue and hard tissue healing responses (26, 44). In contrast, FOXO1 in diabetic conditions has a shift in downstream targets manifested by a reduction in growth factors and an increase in inflammatory mediators that interfere with healing responses (45).

Diabetes also has a significant impact on apoptosis. A mechanism through which diabetes inhibits the early steps of fracture repair is due to the loss of mesenchymal stem cells to apoptosis caused by diabetes-enhanced inflammation (46). The increased apoptosis in bone linked to diabetes is significant since inhibiting apoptosis with a caspase-3 inhibitor or a TNF inhibitor results in significantly enhanced tissue formation in diabetic animals (9, 47, 48). FOXO1 deletion in chondrocytes reversed the effect of diabetes on apoptosis to normal levels. The apoptosis phase of the hypertrophic chondrocytes is a critical phase during fracture repair (2, 40, 49). There is a direct relationship between the vasculature, rate of apoptosis, and resorption of cartilage. It has been proposed that this process is mediated in part

by the tumor necrosis factor alpha (TNF-α). TNF-α enhances the apoptosis of chondrocytes and upregulates the levels of pro-resorptive cytokines that regulate the remodeling phase by osteoclasts (50). Since FOXO1 is downstream of TNF-α, it is likely that activation of FOXO1 in diabetic fracture healing through FOXO1 activation leads to a dysregulation that impairs the healing process. Diabetes significantly upregulated more than 13 apoptotic pathways, including the caspase pathway (Table 3). Our findings here support the concept that FOXO1 upregulates caspase-3 activity in diabetic fractures and that it is transcriptionally regulated by increased FOXO1 binding to the caspase-3 promoter when chondrocytes are exposed to high levels of advanced glycation end products or high glucose levels. Additionally, the transcriptional activity of caspase-3 was directly enhanced by transfection with a FOXO1 expression vector that was significantly enhanced when chondrocytes were incubated in media supplemented with high glucose or AGEs. Apoptosis of chondrocytes may not only limit the formation of the cartilage matrix but lead to greater matrix degradation (51). The increased apoptotic activities may also lead to premature cartilage removal, which has been shown to impair the healing process. And lastly, chondrocyte transdifferentiation into osteoblasts may be inhibited by a loss of chondrocytes through apoptosis, which could ultimately reduce the formation of a bony callus during the endochondral process (52, 53). Based on those observations, the upregulated apoptotic activities can indirectly retard the healing by a number of different mechanisms.

To answer whether FOXO1 activities in chondrocytes represent crucial mechanisms for impaired diabetic long-bone healing, we tested mice with lineage-specific FOXO1 deletion. FOXO1 deletion rescues reduced callus formation caused by diabetes measured by microCT and histologically (3). The mechanical properties of the calluses followed the same pattern. The maximum torque was reduced due to diabetes by ~ 70%, stiffness by 56%, toughness by 74%, and shear modulus by 60%. FOXO1 deletion restored these parameters of mechanical strength to normal levels in diabetic fractures.

In summary, we report here for the first time the important role of FOXO1 and chondrocytes in diabetic fracture healing by inhibiting angiogenesis during the fracture healing process. This contrasts with the positive role that FOXO1 has in promoting angiogenesis in normal animals (31). In addition, we show that FOXO1 in diabetic fracture healing also has a negative effect on increasing chondrocyte apoptosis.

TABLE 3 Apoptosis-related pathways upregulated in diabetic fracture healing.

| Apoptotic gene sets | Upregulated by Diabetes |
|---|---|
| Passerini apoptosis | Yes |
| Apoptosis Kegg | Yes |
| Apoptosis | Yes |
| Death pathway | Yes |
| Vanasse BCL2 targets | Yes |
| NF-kB pathway | Yes |
| TNF and FAS network | Yes |
| TNFA NF-kB dep up | Yes |
| ST FAS signaling pathway | Yes |
| Caspase pathway | Yes |
| PKC pathway | Yes |
| Passerini oxidation | Yes |
| JNK up | Yes |

Gene set enrichment analysis (GSEA) identified apoptosis-related pathways that were significantly upregulated. Original data is found in ref (40).

## Data availability statement

The original contributions presented in the study are included in the article/supplementary files, further inquiries can be directed to the corresponding author/s.

## Ethics statement

The animal study was reviewed and approved by IACUC Committee of the University of Pennsylvania.

## Author contributions

MA: Experimental design, carrying out experiments, data interpretation, writing and revising the manuscript, and preparation and revision of figures. DG: Overall project and experimental design, data interpretation, writing and revising the manuscript, revision of figures, grant funding to support experiments. All authors contributed to the article and approved the submitted version.

## Funding

This work was supported by funding from the NIH grants and R01AR080895. R01DE019108.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note

# References


1. Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *J Cell Biochem* (2003) 88(5):873–84. doi: 10.1002/jcb.10435

2. Lee FY, Choi YW, Behrens FF, DeFouw DO, Einhorn TA. Programmed removal of chondrocytes during endochondral fracture healing. *J Orthop Res* (1998) 16(1):144–50. doi: 10.1002/jor.1100160124

3. Alharbi MA, Zhang C, Lu C, Milovanova TN, Yi L, Ryu JD, et al. FOXO1 deletion reverses the effect of diabetic-induced impaired fracture healing. *Diabetes.* (2018) 67 (12):2682–94. doi: 10.2337/db18-0340

4. Hausman MR, Schaffler MB, Majeska RJ. Prevention of fracture healing in rats by an inhibitor of angiogenesis. *Bone.* (2001) 29(6):560–4. doi: 10.1016/S8756-3282(01) 00608-1

5. Marin C, Luyten FP, van der Schueren B, Kerckhofs G, Vandamme K. The impact of type 2 diabetes on bone fracture healing. *Front Endocrinol (Lausanne).* (2018) 9:6. doi: 10.3389/fendo.2018.00006

6. Street J, Bao M, deGuzman L, Bunting S, Peale FVJr., Ferrara N, et al. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. *Proc Natl Acad Sci U S A.* (2002) 99(15):9656–61. doi: 10.1073/pnas.152324099

7. Sivaraj KK, Adams RH. Blood vessel formation and function in bone. *Development.* (2016) 143(15):2706–15. doi: 10.1242/dev.136861

8. Gibson GJ, Kohler WJ, Schaffler MB. Chondrocyte apoptosis in endochondral ossification of chick sterna. *Dev Dyn.* (1995) 203(4):468–76. doi: 10.1002/aja.1002030409

9. Tanios M, Brickman B, Cage E, Abbas K, Smith C, Atallah M, et al. Diabetes and impaired fracture healing: a narrative review of recent literature. *Curr Osteoporos Rep* (2022) 20(5):229–39. doi: 10.1007/s11914-022-00740-z

10. Guariguata L. Contribute data to the 6th edition of the IDF diabetes atlas. *Diabetes Res Clin Pract* (2013) 100(2):280–1. doi: 10.1016/j.diabres.2013.02.006

11. Hernandez RK, Do TP, Critchlow CW, Dent RE, Jick SS. Patient-related risk factors for fracture-healing complications in the united kingdom general practice research database. *Acta Orthop* (2012) 83(6):653–60. doi: 10.3109/17453674.2012.747054

12. Yamamoto M, Yamaguchi T, Yamauchi M, Kaji H, Sugimoto T. Diabetic patients have an increased risk of vertebral fractures independent of BMD or diabetic complications. *J Bone Miner Res* (2009) 24(4):702–9. doi: 10.1359/jbmr.081207

13. Loder RT. The influence of diabetes mellitus on the healing of closed fractures. *Clin Orthop Relat Res* (1988) (232):210–6. doi: 10.1097/00003086-198807000-00028

14. Kayal RA, Tsatsas D, Bauer MA, Allen B, Al-Sebaei MO, Kakar S, et al. Diminished bone formation during diabetic fracture healing is related to the premature resorption of cartilage associated with increased osteoclast activity. *J Bone Miner Res* (2007) 22(4):560–8. doi: 10.1359/jbmr.070115

15. Kayal RA, Alblowi J, McKenzie E, Krothapalli N, Silkman L, Gerstenfeld L, et al. Diabetes causes the accelerated loss of cartilage during fracture repair which is reversed by insulin treatment. *Bone.* (2009) 44(2):357–63. doi: 10.1016/j.bone.2008.10.042

16. Ivers RQ, Cumming RG, Mitchell P, Peduto AJ. Diabetes and risk of fracture: the blue mountains eye study. *Diabetes Care* (2001) 24(7):1198–203. doi: 10.2337/diacare.24.7.1198

17. Nicodemus KK, Folsom AR. Type 1 and type 2 diabetes and incident hip fractures in postmenopausal women. *Diabetes Care* (2001) 24(7):1192–7. doi: 10.2337/diacare.24.7.1192

18. Vestergaard P, Rejnmark L, Mosekilde L. Relative fracture risk in patients with diabetes mellitus, and the impact of insulin and oral antidiabetic medication on relative fracture risk. *Diabetologia.* (2005) 48(7):1292–9. doi: 10.1007/s00125-005-1786-3

19. Herskind AM, Christensen K, Norgaard-Andersen K, Andersen JF. Diabetes mellitus and healing of closed fractures. *Diabete Metab* (1992) 18(1):63–4.

20. Ward NH3rd, Wainwright DJ. Outcomes research: mandibular fractures in the diabetic population. *J Craniomaxillofac Surg* (2016) 44(7):763–9. doi: 10.1016/j.jcms.2015.09.001

21. Sadhu Reddipogu J, Lightfoot E, Scott C, Thomas M. Recurrent mandibular fractures: a retrospective study over 17 years on aetiology, demographics, fracture patterns, and management. *Int J Oral Maxillofac Surg* (2021) 50(12):1596–602. doi: 10.1016/j.ijom.2021.05.002

22. Ponugoti B, Dong G, Graves DT. Role of forkhead transcription factors in diabetes-induced oxidative stress. *Exp Diabetes Res* (2012) 2012:939751. doi: 10.1155/2012/939751

23. Teixeira CC, Liu Y, Thant LM, Pang J, Palmer G, Alikhani M. Foxo1, a novel regulator of osteoblast differentiation and skeletogenesis. *J Biol Chem* (2010) 285 (40):31055–65. doi: 10.1074/jbc.M109.079962

24. Wang C, Shen J, Ying J, Xiao D, O'Keefe RJ. FoxO1 is a crucial mediator of TGF-beta/TAK1 signaling and protects against osteoarthritis by maintaining articular cartilage homeostasis. *Proc Natl Acad Sci U S A.* (2020) 117(48):30488–97. doi: 10.1073/pnas.2017056117

25. Kurakazu I, Akasaki Y, Tsushima H, Sueishi T, Toya M, Kuwahara M, et al. TGFbeta1 signaling protects chondrocytes against oxidative stress via FOXO1-autophagy axis. *Osteoarthritis Cartilage.* (2021) 29(11):1600–13. doi: 10.1016/j.joca.2021.07.015

26. Ding Z, Qiu M, Alharbi MA, Huang T, Pei X, Milovanova TN, et al. FOXO1 expression in chondrocytes modulates cartilage production and removal in fracture healing. *Bone.* (2021) 148:115905. doi: 10.1016/j.bone.2021.115905

27. Matsuzaki T, Alvarez-Garcia O, Mokuda S, Nagira K, Olmer M, Gamini R, et al. FoxO transcription factors modulate autophagy and proteoglycan 4 in cartilage homeostasis and osteoarthritis. *Sci Transl Med* (2018) 10(428):16–28. doi: 10.1126/scitranslmed.aan0746

28. Waller KA, Zhang LX, Elsaid KA, Fleming BC, Warman ML, Jay GD. Role of lubricin and boundary lubrication in the prevention of chondrocyte apoptosis. *Proc Natl Acad Sci U S A.* (2013) 110(15):5852–7. doi: 10.1073/pnas.1219289110

29. Karamchedu NP, Tofte JN, Waller KA, Zhang LX, Patel TK, Jay GD. Superficial zone cellularity is deficient in mice lacking lubricin: a stereoscopic analysis. *Arthritis Res Ther* (2016) 18:64. doi: 10.1186/s13075-016-0967-4

30. Shen Q, Xiao Y, Cheng B, Sun Z, Hu Y, Yang H, et al. PRMT1 promotes extracellular matrix degradation and apoptosis of chondrocytes in temporomandibular joint osteoarthritis via the AKT/FOXO1 signaling pathway. *Int J Biochem Cell Biol* (2021) 141:106112. doi: 10.1016/j.biocel.2021.106112

31. Zhang C, Feinberg D, Alharbi M, Ding Z, Lu C, O'Connor JP, et al. Chondrocytes promote vascularization in fracture healing through a FOXO1-dependent mechanism. *J Bone Miner Res* (2019) 34(3):547–56. doi: 10.1002/jbmr.3610

32. Lu Y, Alharbi M, Zhang C, O'Connor JP, Graves DT. Deletion of FOXO1 in chondrocytes rescues the effect of diabetes on mechanical strength in fracture healing. *Bone.* (2019) 123:159–67. doi: 10.1016/j.bone.2019.03.026

33. Ovchinnikov DA, Deng JM, Ogunrinu G, Behringer RR. Col2a1-directed expression of cre recombinase in differentiating chondrocytes in transgenic mice. *Genesis.* (2000) 26(2):145–6. doi: 10.1002/(SICI)1526-968X(200002)26:2<145::AID-GENE14>3.0.CO;2-C

34. Graves DT, Naguib G, Lu H, Leone C, Hsue H, Krall E. Inflammation is more persistent in type 1 diabetic mice. *J Dent Res* (2005) 84:324–8. doi: 10.1177/154405910508400406

35. Like AA, Rossini AA. Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. *Science.* (1976) 193(4251):415–7. doi: 10.1126/science.180605

36. Gerstenfeld LC, Wronski TJ, Hollinger JO, Einhorn TA. Application of histomorphometric methods to the study of bone repair. *J Bone Miner Res* (2005) 20 (10):1715–22. doi: 10.1359/JBMR.050702

37. Mirando AJ, Dong Y, Kim J, Hilton MJ. Isolation and culture of murine primary chondrocytes. *Methods Mol Biol* (2014) 1130:267–77. doi: 10.1007/978-1-62703-989-5_20

38. Rozovski U, Harris DM, Li P, Liu Z, Wu JY, Grgurevic S, et al. At High levels, constitutively activated STAT3 induces apoptosis of chronic lymphocytic leukemia cells. *J Immunol* (2016) 196(10):4400–9. doi: 10.4049/jimmunol.1402108

39. Lim JC, Ko KI, Mattos M, Fang M, Zhang C, Feinberg D, et al. TNFalpha contributes to diabetes impaired angiogenesis in fracture healing. *Bone.* (2017) 99:26–38. doi: 10.1016/j.bone.2017.02.014

40. Kayal RA, Siqueira M, Alblowi J, McLean J, Krothapalli N, Faibish D, et al. TNF-alpha mediates diabetes-enhanced chondrocyte apoptosis during fracture healing and stimulates chondrocyte apoptosis through FOXO1. *J Bone Miner Res* (2010) 25 (7):1604–15. doi: 10.1002/jbmr.59

41. Jiao H, Xiao E, Graves DT. Diabetes and its effect on bone and fracture healing. *Curr Osteoporos Rep* (2015) 13(5):327–35. doi: 10.1007/s11914-015-0286-8

42. Burgess JL, Wyant WA, Abdo Abujamra B, Kirsner RS, Jozic I. Diabetic wound-healing science. *Medicina (Kaunas).* (2021) 57(10):51–61. doi: 10.3390/medicina57101072

43. Lu C, Miclau T, Hu D, Marcucio RS. Ischemia leads to delayed union during fracture healing: a mouse model. *J Orthop Res* (2007) 25(1):51–61. doi: 10.1002/jor.20264

44. Jeon HH, Yu Q, Lu Y, Spencer E, Lu C, Milovanova T, et al. FOXO1 regulates VEGFA expression and promotes angiogenesis in healing wounds. *J Pathol* (2018) 245 (3):258–64. doi: 10.1002/path.5075

45. Ko KI, Sculean A, Graves DT. Diabetic wound healing in soft and hard oral tissues. *Transl Res* (2021) 236:72–86. doi: 10.1016/j.trsl.2021.05.001

46. Ko KI, Syverson AL, Kralik RM, Choi J, DerGarabedian BP, Chen C, et al. Diabetes-induced NF-kappaB dysregulation in skeletal stem cells prevents resolution of inflammation. *Diabetes.* (2019) 68(11):2095–106. doi: 10.2337/db19-0496

47. Pacios S, Andriankaja O, Kang J, Alnammary M, Bae J, de Brito Bezerra B, et al. Bacterial infection increases periodontal bone loss in diabetic rats through enhanced apoptosis. *Am J Pathol* (2013) 183(6):1928–35. doi: 10.1016/j.ajpath.2013.08.017

48. Ko KI, Coimbra LS, Tian C, Alblowi J, Kayal RA, Einhorn TA, et al. Diabetes reduces mesenchymal stem cells in fracture healing through a TNFalpha-mediated mechanism. *Diabetologia.* (2015) 58(3):633–42. doi: 10.1007/s00125-014-3470-y

49. Li G, White G, Connolly C, Marsh D. Cell proliferation and apoptosis during fracture healing. *J Bone Miner Res* (2002) 17(5):791–9. doi: 10.1359/jbmr.2002.17.5.791

50. Gerstenfeld LC, Cho TJ, Kon T, Aizawa T, Tsay A, Fitch J, et al. Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption. *J Bone Miner Res* (2003) 18(9):1584–92. doi: 10.1359/jbmr.2003.18.9.1584

51. Komatsu DE, Bosch-Marce M, Semenza GL, Hadjiargyrou M. Enhanced bone regeneration associated with decreased apoptosis in mice with partial HIF-1alpha deficiency. *J Bone Miner Res* (2007) 22(3):366–74. doi: 10.1359/jbmr.061207

52. Bahney CS, Hu DP, Taylor AJ, Ferro F, Britz HM, Hallgrimsson B, et al. Stem cell-derived endochondral cartilage stimulates bone healing by tissue transformation. *J Bone Miner Res* (2014) 29(5):1269–82. doi: 10.1002/jbmr.2148

53. Zhou X, von der Mark K, Henry S, Norton W, Adams H, de Crombrugghe B. Chondrocytes transdifferentiate into osteoblasts in endochondral bone during development, postnatal growth and fracture healing in mice. *PloS Genet* (2014) 10 (12):e1004820. doi: 10.1371/journal.pgen.1004820

# Frontiers in
# Dental Medicine

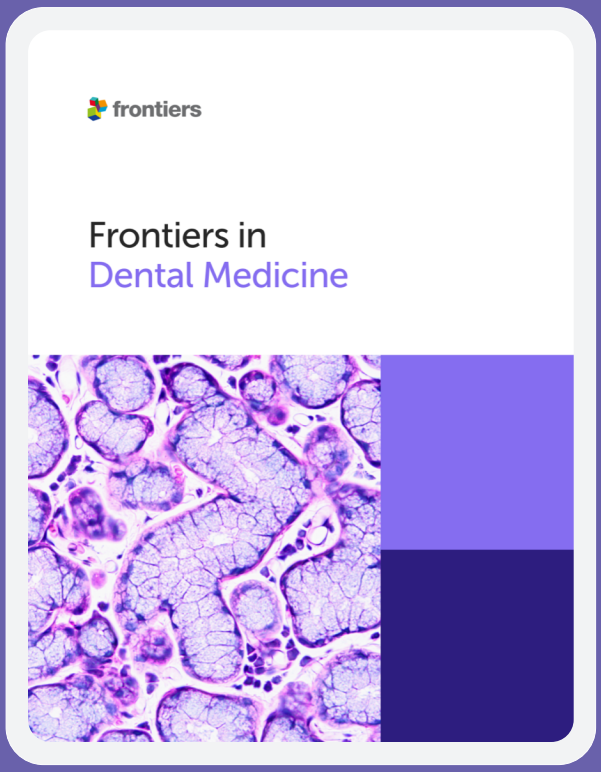
**Explores how dental health and disease impacts overall health**

An interdisciplinary journal that investigates how dental, oral and craniofacial health and diseases are understood in the context of the whole body. Its goal is to improve oral and overall health outcomes for all communities.

---

## Discover the latest Research Topics

See more →




**Frontiers**

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

**Contact us**

+41 (0)21 510 17 00
frontiersin.org/about/contact