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Recent Advances in the Use of Serological Bone Formation Markers to Monitor Callus Development and Fracture Healing

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Abstract

The failure of an osseous fracture to heal, or the development of a nonunion, is common; however, current diagnostic measures lack the capability of early and reliable detection of such events. Analyses of radiographic imaging and clinical examination, in combination, remain the gold standard for diagnosis; however, these methods are not reliable for early detection. Delayed diagnosis of a nonunion is costly from both the patient and treatment standpoints. In response, repeated efforts have been made to identify bone metabolic markers as diagnostic or prognostic tools for monitoring bone healing. Thus far, the evidence regarding a correlation between the kinetics of most bone metabolic markers and nonunion is very limited. With the aim of classifying the role of biological pathways of bone metabolism and of understanding bone conditions in the development of osteoporosis, advances have been made in our knowledge of the molecular basis of bone remodeling, fracture healing, and its failure. Procollagen type I amino-terminal propeptide has been shown to be a reliable bone formation marker in osteoporosis therapy and its kinetics during fracture healing has been recently described. In this article, we suggest that procollagen type I amino-terminal propeptide presents a good opportunity for early detection of nonunion. We also review the role and potential of serum PINP, as well as other markers, as indications of fracture healing.

Keywords

procollagen type I amino-terminal propeptide (PINP); fracture healing; non-union; collagen synthesis; bone metabolic marker; bone turnover marker

I. INTRODUCTION

In 2005, 13.9% of the 115.3 million emergency department visits in the United States were related to musculoskeletal disorders.¹ Long-bone fractures are common and are occurring with increasing frequency.^{2–4} Repair and regeneration of bony injuries are complex processes requiring interaction of mesenchymal cells at different stages of differentiation and a coordinated cascade of growth factors and cytokines. The end result is the production

of extracellular matrix, which is eventually converted into bone. The extracellular matrix consists of approximately 90% collagen type I and is first formed in the callus. Subsequently, intercellular and intracellular signaling regulates transcriptional and translational transformation of the callus from a transient cartilaginous matrix into calcified bone.⁵

Clinically, fracture healing is defined as being delayed if the healing process has not been completed within 3 to 6 months.⁶ The US Food and Drug Administration defines failure of fracture healing, or development of a nonunion, to occur after a minimum of 9 months from the date of fracture with no radiographic signs of progression toward healing for 3 consecutive months.⁷ The development of a nonunion is a common complication following long-bone fracture. Its incidence ranges up to 46%,^{6,8–12} depending on injury location, bone loss, soft tissue injury, and associated vascular injuries. Other risk factors for nonunion include: fracture type,¹³ surgical technique,¹⁴ infection,¹⁵ and patient-related factors such as age,⁹ co-morbidities,^{16–18} tobacco use,^{19,20} diet, and alcohol consumption.¹⁷

To date, no clinical or scientific assessment can reliably predict successful fracture healing or nonunion. The diagnosis of a nonunion is typically made by attentive clinical examination. Patients with a nonunion complain of persistent pain of the involved bone during weight bearing and passive range of motion due to fracture site instability.²¹ Radiographic imaging is an adjunct tool that typically shows persistent fracture site lucency.⁶ Radiographic scoring has been helpful, but not perfect, in predicting fracture healing.²² However, computerized tomography with reconstructed views still seems to be the most useful non-operator-dependent method for the evaluation of delayed or failed long-bone union.²³

The current problem of nonunion is that the delayed diagnosis leads to months of pain, suffering, physical therapy, and inability to perform activities of daily living, and may often lead to a patient's unemployment. The prevention of a nonunion by an intervention is not possible after waiting for months to diagnose the mature nonunion. An early intervention by changing conservative management or initiating surgical intervention could prevent further complications,^{24,25} prolonged patient distress, and disability.²⁶ Early intervention with limited and less invasive methods of treatment could also lessen the morbidity and costs of current nonunion surgery. Furthermore, such an early intervention could shorten the patient's recovery time, resulting in a significant impact on health care system costs and society.^{27,28}

This review discusses the molecular physiology and pathophysiology of bone healing; particular attention is paid to the role of collagen type I synthesis in callus formation and responses of bone turnover markers during healing. The ultimate goal is to review the potential of such markers for early diagnosis of a nonunion.

II. MOLECULAR BASIS OF COLLAGEN SYNTHESIS

Since the early description of collagen structure,²⁹ the understanding of collagen synthesis has continually improved and its molecular basis of function has been further characterized.^{30,31} This process is summarized in Fig. 1.

Fibrillogenesis, or cartilage fibril formation, is an entropy-driven assembly process.³² Evidence of the existence of N-terminal extensions of collagen is derived from the observation that procollagen, the intracellular precursor of extracellular collagen, has an approximately 20% higher molecular weight than extracellular collagen.^{33,34} The cleavage of procollagen by specific NH₂-terminal and COOH-terminal proteases has been demonstrated *in vitro*^{35,36} and *in vivo*.^{37,38} Bovine tendon analyses suggest that intact type I

procollagen N-proteinase is an enzyme with a molecular weight of 500 kDa that is involved in the processing of type I procollagen.³⁹ This 500-kDa complex contains polypeptide chains of 161 and 135 kDa that are catalytically active. The sensitivity of the N-proteinase to the conformation of procollagen has been related to some important biological functions of collagen.⁴⁰ The proteinase serves as a quality checkpoint for monomers that have a correct N-terminal conformation and thus can self-assemble into tightly packed fibrils.⁴⁰

In bone, collagen type I forms a three-dimensional framework, which is a triple helical molecule composed of two $\alpha 1$ polypeptide chains and one $\alpha 2$ chain.⁴¹ The triple helical molecules are staggered and contain gaps between the NH₂-terminus of one triple helical molecule and the COOH-terminus of the next. Hexagonal packing of the molecules has been proposed in which the holes are contiguously arranged in one direction.⁴² A specific cross-link in the COOH-terminal region was found to link $\alpha 1$ chains to $\alpha 2$ chains, and has been found to be important for the ordered formation of three-dimensional collagen type I structures.^{41,43} Pyridinoline and deoxypyridinoline are covalent pyridinium cross-links formed by the lysyl oxidase-mediated system during collagen maturation.⁴⁴ These crosslinks are characterized by enzymatic proteolytic resistance and are released into the circulation during collagen degradation.⁴⁵ Osteoglycin, a small leucine-rich proteoglycan, regulates collagen type I fibrillogenesis, which is accelerated by BMP-1.⁴⁶

Helseth and Veis provided evidence that both NH₂-terminal and COOH-terminal telopeptides are important during aggregation to form collagen fibrils.³² Although the NH₂-terminal telopeptide is more important in the initial nucleation or fibril growth priming, the COOH-terminal telopeptide has been thought to regulate the growth reaction. Functionally, it has been proposed that the N-propeptides of collagen type I form stable helical structures, control collagen fibrillogenesis,⁴⁷ and regulate feedback control mechanisms of collagen biosynthesis.⁴⁸ Evidence suggests that NH₂-peptides are responsible for the intracellular assembly of the three procollagen chains.³⁵ Furthermore, procollagen type I amino-terminal propeptide (PINP) has been attributed a role as a temporary blocking agent of the mineralization within the “gap regions” of the collagen fibril, which is initiated by removal of the N-propeptide. It is also thought to be a bioactive bone matrix marker for bone-resorbing cells.⁴⁹

Mutations near the NH₂-terminal end of the 1 chain of collagen type I are known to cause genetic disorders, such as osteogenesis imperfecta and Ehlers-Danlos syndrome, by interfering with the processing of the NH₂-terminus of procollagen type I.⁵⁰ Bone fragility and growth deficiency characterize these connective tissue disorders.^{51,52}

III. COLLAGEN ACTIVITY IN FRACTURE HEALING

The healing of fractured bone is a complex and unique regenerative process that reestablishes former function and form. Messenger RNA analysis in rat femoral fracture models found age-independent up-regulation of approximately 1200 genes and down-regulation of 900 genes involved in fracture repair.⁵³ These processes are persistent biomolecular events reminiscent of embryonic skeletal development.⁵⁴ However, unlike the embryological phases of bone development (including phasic cycling of bone formation), fracture repair follows a one-way path of similarly regulated chondrogenic and osteoblastic phases of bone formation. Similar to liver tissue, bone has the potential of “restitutio ad integrum,” in contrast to most other tissues, which tend to heal with scar formation. Scars lack the characteristics of the former structure not only in form (cellular and intercellular structure), but also in function, biomechanics, and cellular and intercellular physiological processing. Bone is a composite and the organic content of the extracellular matrix of human bone consists of approximately 90% of collagen type I,^{55,56} which makes it the most

abundant protein in humans. It maintains the integrity of tissues by mediating interactions with cell surfaces and other extracellular matrix molecules and by facilitating signaling by growth and differentiation factors.⁵⁷ Although collagen type I is predominant in bone, collagen type II is found in cartilage and collagen type III in soft tissue.⁵⁸ The spatial and temporal sequences of collagen synthesis during fracture repair are not well understood.

During fracture healing, both endochondral and intramembranous ossification can occur, often simultaneously. With intermediate strain, endochondral ossification results in externally formed bone, often in close relation to the periosteum.⁵⁹ Intramembranous bone formation occurs on the inner layer of the periosteum at the proximal and distal fracture edges, forming the hard fracture callus.⁶⁰ On the histological level, fracture healing⁶¹ and endochondral ossification^{62,63} have been well described. As shown in Fig. 2, fracture healing is characterized by three phases: inflammation (days 1–3), reparation (chondrogenic, days 5–10), and remodeling (osteogenic, days 14–21).⁶¹ In recent years, these phases have been further characterized on the transcriptional level.^{64,65}

The fracture healing process is dominated by bone formation during the first two phases.⁶⁶ During the inflammation phase, the external callus is confined in a fibrous capsule consisting mainly of unorganized granulation tissue.⁶⁴ Page et al. demonstrated that in mechanically stable as well as in unstable fractures, mesenchymal cells secrete a collagenous matrix that consists predominantly of collagen type III.⁵⁹ Periosteal thickening and cell proliferation have been observed.⁶⁷ The inflammation phase is further characterized by the invasion of mesenchymal cells that differentiate into chondrocytes for cartilage formation and osteoblasts for bone formation. Initial cell debris and hematoma are replaced by fibrous tissues, which have been found to be collagen type III.⁵⁹ During this early stage of fracture healing, Page et al. showed that small amounts of collagen type I can be found in isolated areas within the callus adjacent to the cortical bone, which represents the first stages in the development of cancellous bone trabeculae.⁵⁹ Similarly, Lane et al. noted that the periosteum forms a primitive mesenchymal matrix (consisting of collagen type I and III) and during the early phase undergoes a transition from metaplastic to membranous bone formation.⁶³ The amount of collagen type I fibers increases until day 5 after fracture, but the predominant fiber type consists of collagen type III.⁵⁹

The reparation phase has been attributed to intramembranous bone formation originating from the periosteal region.⁶⁷ Vascular invasion and callus growth, with a peak at day 14, characterize the reparation phase. Histomorphological data indicate a presence of collagen type I, III, and V.⁵⁹

The remodeling phase has been characterized as endochondral trabecular bone formation associated with osteoblast and TRAP-positive cell settlement in the marrow cavity, unity of fragment ends, and regeneration of bone marrow space.⁶⁷ These events agree with experimental data from rabbit fracture models showing increasing amounts of trabecular bone with predominance of collagen type I, but with the persistent presence of collagen type III and V in the central regions of the trabeculae.⁵⁹ From their experimental work on endochondral ossification in rat tibial fractures, Lane et al. concluded that fracture healing with decreased mechanical strain or anoxia is characterized by a chondroid transformation of the matrix as a prerequisite to lamellar bone formation, whereas a mesenchymal-to-lamellar bone transition proceeds in proximity to the periosteum with minor motion and preserved blood supply.⁶³

Sandberg et al. measured the cellular levels of specific mRNAs using in situ hybridization to facilitate advances in the determination of when and where a given type of collagen is being synthesized.⁶⁴ Within the first week of fracture repair, the callus can be identified,

consisting of unorganized granulation tissue with early signs of cartilage matrix adjacent to cortical bone and appositional new woven bone growth. However, recent data demonstrate that osteogenesis is induced in the early phase following fracture, represented by a strong induction of type I collagen mRNA expression.⁶⁸ Cartilaginous callus continues to grow during the second week of fracture repair and begins to be replaced by woven bone. By 3 to 4 weeks postfracture, most of the cartilage matrix has been replaced by bone. In rat femoral-defect models, collagen type I mRNA has been detected in osteoblastic cells on the marrow side of the hematoma during the first days of the inflammation phase.⁶⁶

A dynamic pattern of collagen type I expression has been described during the phases of fracture healing. A marked increase in the level of collagen type I mRNA is measured during the first 7 days. The levels of pro-1(I) collagen mRNA peak at 1 week in osteoblasts lining the trabeculae of woven bone. The levels of pro α -1(I) collagen mRNA decline by day 28.⁶⁴ Similar characterizations of fracture repair reveal a sequential predominance in spatial and temporal collagen type I expression and maximum expression of pro α -1(III) at 5 days, of pro α -1(II) at 10 days, of pro α -1(I) at 14 to 28 days, and of pro α -2(I) at 14 days.⁶⁵ Other authors observed low levels of pro α 1(I) collagen mRNA in normal bone.⁶⁹ Kon et al. demonstrated that the formation of intramembranous bone in the periosteum during the first 2 weeks of fracture healing is associated with increased levels of mRNAs for collagen type I and osteocalcin.⁶⁸ Finally, the expression of collagen type I mRNA, in conjunction with MMP-13 mRNA expression by immature osteoblastic cells during the reparation phase, has been recognized as marking the initiation of bone remodeling.⁶⁷ Collagen type I mRNA is most abundant in osteoblasts lining the osseous trabeculae,⁶⁶ but it has also been observed in fibrous callus and in chondrocytes along the margin of the expanding cartilage.⁶⁴

Within the extracellular matrix, the cell-to-collagen type I interaction is mediated by specific integrins, which are composed of four heterodimers (α 1, α 2, α 10, or α 11, including a collagen type I selective binding domain) and a common β 1 subunit. Integrin α 1 β 1 has been shown to inhibit collagen type I synthesis, whereas integrin α 2 β 1 can induce collagen type I gene expression.^{70–72} The expression of these integrins is regulated by TGF- β .⁷¹

Endochondral ossification is dependent on matrix remodeling proteinases for the degradation of cartilage into bone. Matrix metalloproteinases (MMPs) are key enzymes in the physiological degradation of extracellular matrix of bone, cartilage, and other soft tissues. Most MMPs are secreted into the intercellular matrix; however, membrane-type MMPs can be membrane-bound⁷³ and are responsible for skeletal transformation resulting in long-bone maturation via regulation of cell proliferation, protein degradation, and cellular activity.⁷⁴ MMP-1 has been linked to the processing of collagen type I.⁷⁵

IV. CAUSES AND CHARACTERISTICS OF FAILED FRACTURE HEALING RELATED TO COLLAGEN

To classify nonunions, we have traditionally used a schema based on the amount of callus or bone healing at the fracture site. The development of a nonunion is the end point represented by cessation of periosteal and endosteal new bone formation, including medullary sclerosis of the former fracture ends. According to Marsh, failed fracture healing is defined as a process of scar formation with zero or low endosteal and periosteal osteogenic activity, as shown radiographically in Fig. 3A to 3C.⁷⁶ Hypertrophic nonunions show prolific callus formation without fracture gap closure and are typically attributed to a defect in stabilization.⁷⁷ These have the vascular and biological ability to heal but have too much strain or instability, preventing healing. Oligotrophic nonunions show some callus formation.⁷⁷ Atrophic nonunions are characterized by an absence of callus formation and an elongated fracture gap.⁷⁷ Atrophic and oligotrophic nonunions have been traditionally

thought to be deficient in the biological ability to heal. Segmüller et al. provided evidence from scintigraphic analysis of atrophic nonunions that biologically inactive nonunions are rare and that atrophic, low-callus-forming nonunions have good vascularization and biological activity.⁷⁸ These early observations have been supported by recent investigations.⁷⁹ Thus, a defect in healing can be considered as either primarily biologic (multifactorial), biomechanical (frequently implant-related or alignment-related), or mechanical (almost always implant-related).

Temporal and spatial collagen type I distribution has been immunohistochemically determined in an experimental rat nonunion model.⁸⁰ In the early phase, at 1 week, collagen type I was localized subperiosteally in close proximity to lamellar structured new bone. Thereafter, collagen type I was identified at newly formed lamellar periosteal bone, as well as at the endochondral ossification front. After 5 weeks, collagen type I was the most prevalent between the lamellar bone and fibrous callus sleeve. With initiation of the remodeling phase, collagen type I was quantitatively decreased, reflecting a diminished expression rate. However, collagen type I remained present in the fibrous callus until week 9, after which collagen type I could not be detected within the nonunion.

Brick et al. observed peak gene expression of collagen type I at 2 weeks in an infected segmental defect model, which was comparable to other regenerative processes of bone (i.e., de novo osteogenesis, fracture healing, bone remodeling).⁸¹ Increased collagen type I expression under uninfected conditions (compared with recombinant human bone mineral protein 2 [rhBMP-2] treatment) was seen in the early phase, indicating bone matrix formation. On the other hand, rhBMP-2 did maintain collagen type I mRNA up-regulation despite infection for more than 4 weeks. Brick et al. hypothesized that an infected segmental defect BMP is able to accelerate remodeling, inter alia, by increased collagen type I synthesis.

The presence of an osteoblast phenotype expressing the gene for matrix GLA protein (osteocalcin) has been observed in nonunions, indicating an abnormal gene expression in human nonunions.⁸² In this context, significant procollagen type I mRNA expression has been evidenced in osteoblasts on woven bone in human nonunions and normal fractures.⁸³ Interestingly, prolonged expression of collagen type III mRNA was recognized in woven bone near osteoblasts adjacent to the nonunion site. If the repair process was impaired by instability, other findings indicate a time-limited healing potency of a fracture.⁸⁰ The normal healing process stops and matrix mineralization of the callus is delayed, leading to production of fibroblasts, collagen type III, and fibronectin. Thus, production of collagens and other matrix components is initiated and leads to healing with scar tissue and nonunion.

The activity of growth and differentiation factors can be modulated by components of the extracellular matrix. In this context, collagen type I has been shown to bind to and potentiate the activity of BMP-2b, a member of the TGF- β superfamily.⁸⁴ In humans, MMPs have also shown different kinetics in delayed fracture healing compared with normal healing.⁸⁵ Within an 8-week period, a significant difference in the presence of systemically detectable MMPs has been described during long-bone fracture healing.⁸⁶ At 8 weeks, no further differences in the kinetics of immunochemically traceable MMPs in venous blood were apparent. Similarly, in pig femoral-defect-regeneration models, immunohistochemical differences of MMPs in bone defect regeneration supplemented by vancomycin-loaded allografts have been reported.⁸⁷ Waters et al. demonstrated that chronic corticosteroid administration impairs non-critical-sized ulnar fracture healing in rabbits, which might be related to corticosteroidal reduction of collagen type I and osteocalcin mRNA synthesis.⁸⁸ In this context, Lu et al. provided evidence from MC3T3-E1 cell culture studies that glucocorticoids inhibit TGF- β -induced collagen type I expression.⁸⁹

Other influencing factors have been projected from mechano-regulation modeling systems used to predict tissue differentiation and organization during fracture repair. Nagel et al. concluded that collagen organization within a fracture might be regulated by the local mechanical environment.⁹⁰ External callus was dominated by uniformly aligned fibers during stress-driven remodeling, which was hypothesized to contribute to fracture stabilization by supporting tensile stresses during bending. Most recently, nicotine has been found to influence collagen type I expression in osteoblast cell studies.⁹¹ After an initial up-regulation of type I collagen gene expression (approximately 140%) during the first 24 hours of incubation, a dramatic down-regulation of collagen type I mRNA gene expression by nicotine—in a dose-dependent manner, after 48 and 72 hours of incubation—was observed. This resulted in a decrease in cell proliferation and, ultimately, in cell death. Interestingly, no change in type I collagen expression was observed after short-term (less than 6 hours) exposure to nicotine.

V. SEROLOGICAL MARKERS OF BONE METABOLISM AND REGENERATION

Bone biopsy is the most accurate measure of bone disease and bone turnover dynamics because it makes possible a direct quantitative and functional determination of cells, an analysis of cortical and cancellous structural changes, and an evaluation of bone turnover dynamics. However, this procedure is invasive and expensive, and it can result in pain or infection at the biopsy site. Because serum bone markers are obtained in a less invasive fashion and are less expensive but still able to assess bone metabolism and diseases, commercially available biochemical serum bone markers may be more advantageous. Efforts to establish a serological indicator for fracture healing and bone metabolism date from the early 1980s,^{92–94} although fracture-related alkaline phosphatase dynamics have been investigated since the early 1950s.^{95,96} The measurement of bone turnover markers (BTMs) in serum quantitatively assesses the rate of bone remodeling; therefore, this method could be a valid tool for monitoring changes of bone formation. The markers can represent bone formation, matrix breakdown, and enzymatic activity of bone cells.⁹⁷ Bone formation (but not bone resorption) markers have been correlated with physical activity.⁹⁸ Some authors have observed that bone formation markers are not suitable for detecting an increase of hard callus tissue, thus indicating increased fracture stability.⁹⁹ Bone remodeling and regeneration, the lifelong processes of renewal of collagen and mineral structures, are promoted by a well-balanced interaction of bone resorption and formation. Although there is some overlap in bone formation and bone resorption markers, we focus on bone formation markers in this review because they are the most germane to bone healing. While some bone resorption does occur early after fracture, healing cannot proceed to completion without new bone formation.

VI. BONE FORMATION MARKERS

Serological markers monitoring matrix degradation during bone formation are predominantly derived from osteoblasts. Current serum markers of bone formation activity include bone-specific alkaline phosphatase (ALP), procollagen type-I N-terminal propeptide (PINP), procollagen type-I C-terminal propeptide (PICP), and osteocalcin (OC).

A. Alkaline Phosphatase

One marker elevated in all mineralizing tissues is ALP, an ectoenzyme of osteoblasts capable of generating extracellular inorganic phosphate. ALP is found in the plasma membrane of osteoblasts.¹⁰⁰ High ALP levels and an increased rate of matrix vesicle production are found in cartilage prior to matrix calcification.^{101,102} An increased specificity

of monoclonal antibodies for small differences in the molecular structures of antigens forms the basis of further differentiation and specification of ALP isoenzymes among tissues. ALP activity has been verified in kidney, intestine, and placenta tissues, but significant serum secretion has been linked to bone and liver tissues.¹⁰³ The bone-specific isoenzyme contributes a specific amount to total serum ALP, which can now be routinely measured.¹⁰⁴ ALP assays are characterized by minimal diurnal variation.¹⁰⁵ Expression of ALP mRNA is highest 10 to 14 days after fracture in fibrous tissue cells adjacent to newly formed bone, as well as in osteoblasts on the surface of newly formed trabecular bone, but not in the hematoma during the inflammation phase of fracture healing.⁶⁶

Conditions such as osteomalacia and vitamin D deficiency result in increased levels of ALP despite decreased bone formation.¹⁰⁶ Evidence has been provided for the stimulating effect of vitamin D₃ and its metabolites (1,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol) on ALP expression in osteoblasts in vitro and in vivo.^{107–109} Furthermore, a cross-reactivity of approximately 15% between liver and bone ALP can affect measurements in individuals with high liver ALP.¹⁰³

B. PINP and PICP

Type I collagen is cross-linked at the N-terminal and C-terminal ends, providing the bone with its basic fabric and tensile biomechanical properties.¹¹⁰ As shown in Fig. 4, bone collagen is derived from type I procollagen, which has propeptide extensions at both ends of the molecule that are removed by specific proteinases before the molecules are assembled into collagen fibrils.^{56,111} When the propeptides are removed, a measurable concentration of them can be found in the blood. The serum concentration of PINP is directly proportional to the amount of new collagen produced by osteoblasts.⁵⁶ Intact PINP is a partially collagenous, phosphorylated protein with a mass of 35 kDa.¹¹² Unlike collagen type III, N-terminal cleavage in collagen type I formation is complete.¹¹³ PINP is cleared from the circulation by scavenger receptor-mediated endocytosis in sinusoidal liver endothelial cells.¹¹⁴

Han et al. showed in rats that BTMs can have similar trends, but that PINP had more significant changes in concentration than ALP or OC under conditions of altered bone formation rates.¹¹¹ PINP is primarily released during the bone formation process and is therefore more specific for this process than OC.¹¹⁵ Gender-specific differences in serum concentrations have been shown for PINP, with ranges of 20 to 76 µg/L for men and 19 to 84 µg/L for women.¹¹² Furthermore, an age-dependent level of secretion has to be considered; for example, in persons with an immature skeleton, a 20-fold higher level of PINP has been reported.¹¹¹ PINP concentrations are determined by bone formation rather than by procollagen peptide degradation and clearance,⁵⁶ although evaluation of bone formation by PINP is limited by measurement of procollagen fragments, which are not solely released from bone.¹¹⁶ In contrast to bone metabolism parameters, PINP has not been found to be associated with the level of physical activity.⁹⁸

PINP is now proposed for use as a BTM in metabolic bone diseases such as osteoporosis,^{117–124} and has been used to monitor bone turnover in osteoporosis therapy.^{117,123,125–129} However, PINP has not been yet approved by the FDA as a BTM for treatment or monitoring of osteoporosis in the United States. As a marker of bone turnover during osteoporosis, PINP is well studied in the rat animal model.^{111,115,130,131} It is also used in the monitoring of Paget's disease^{131–133} and as a bone metastasis marker.^{134–138}

Many studies have also been directed to the evaluation of C-terminal PICP. A good correlation of PICP with ALP and OC has been reported.⁵⁶ Linkhart et al. also showed that although a good correlation between PINP and PICP was present in children, a correlation

could not be proven in adults.⁵⁶ In earlier studies Joerring and Jensen observed an initial decrease in PICP levels on day 4 after hip arthroplasty, followed by an increase until day 14.¹³⁹ These authors concluded that PICP-level kinetics reflected the healing process of the soft tissue and that bone would only contribute less in that experimental setup. However, Eriksen et al. provided evidence for the reliability and specificity of PICP as a marker of cancellous bone formation in metabolic bone disease by showing a correlation of serum concentration of PICP with dynamic bone histomorphometry.¹⁴⁰

C. Osteocalcin

OC, or γ -carboxyglutamic acid-containing protein, is a 6-kDa protein secreted as a 10-kDa precursor from osteoblasts, osteocytes, and odontoblasts.¹⁴¹ OC is the most abundant noncollagen protein of the extracellular bone matrix. Three vitamin K-dependent γ -carboxyglutamic acid residues determine its calcium affinity.¹⁴² The majority of the processed OC peptide is incorporated into bone matrix, and the measured fraction in serum is related to the rate of bone formation.¹⁴³ Unlike PINP, OC is not strictly released during bone formation and therefore OC is less representative of bone formation.¹¹⁵ For example, because OC is partly incorporated into bone matrix, the measured fraction in serum can also be derived from OC released from the bone matrix during resorption.¹⁴⁴ Also unlike PINP, OC has serum levels characterized by diurnal variation due to rapid renal glomerular filtration; thus, a large fraction can be measured in urine.¹⁴⁵ OC has a short half-life of approximately 20 minutes in serum,¹⁴⁶ is subject to circadian rhythms,¹⁴⁷ and undergoes degradation during systemic circulation and sample handling.¹⁴⁸ Furthermore, the levels of mobilization, vitamin D status, and renal disease can influence the serum concentration.^{146,149,150} Despite these potential shortcomings, OC has been validated for in vitro^{141,150–152} and in vivo analysis of human bone metabolism and regeneration,^{98,109,146,147,153} as well as for small^{92,130,154–156} and large^{99,157} animal models.

VII. BONE FORMATION MARKERS AS SEROLOGICAL MARKERS IN DIFFERENT STAGES OF BONE HEALING

Controversy exists about the usefulness of bone formation markers to indicate the state of fracture healing. For example, OC serum levels after fracture have been proposed as useful measures to monitor fracture healing, whereas ALP was not found to be indicative.⁹³ Observations of OC and ALP have shown a positive correlation at injury and 6 weeks after fracture, but have shown no correlation at 12 weeks.¹⁵⁸ However, persistently increasing measures of OC and ALP were thought to reflect increases in osteoblastic activity during delayed tibial diaphyseal fracture healing. With uneventful and successful tibial fracture healing, Kurdy showed a continuous increase up to 20 weeks in bone-specific ALP, after an initial decrease during the first week after fracture.¹⁵⁹ Increasing bone-specific and total ALP measurement in sheep fracture models reflected an increase in bone formation from the fourth week posttrauma onward, but due to large inter-individual variation, its general suitability as a measure of healing has been questioned.⁹⁹

Lotz et al. characterized collagen type I metabolism in patients after uncomplicated bone surgery and observed different PICP concentrations depending on the type of intervention.¹⁶⁰ Callus-mediated bone healing led to higher PICP concentrations, which was interpreted as a reflection of strong collagen type I synthesis. PICP serum levels can correlate with age during normal fracture healing in humans.¹⁶¹ In an earlier study, Joerring et al. showed that after long-bone fracture, PICP concentration increased at 1 week and reached its maximum at 2 weeks, followed by a decline thereafter.¹⁶² The treatment type (cast immobilization vs. osteosynthesis with plates and screws) produced no kinetic

difference in PICP. Interestingly, a higher relative increase in PIIINP versus PICP was found during the first 2 weeks of fracture healing, reflecting a more pronounced collagen type III synthesis, which is in accordance with immunohistological findings from animal fracture models.^{59,63} Contrarily, kinetics similar to that described for bone-specific ALP has been observed for PICP serum concentrations.¹⁵⁹ Kurdy et al. reported findings from normal healing tibial fractures that showed a significant drop in PICP levels between days 4 through 8, followed by an increase by days 14 to 20,¹⁶¹ which was related to a temporary cessation of osteoblast activity in response to a fracture.

OC levels in animal fracture models displayed small temporal and inter-individual variation.⁹⁹ Obrant et al. reported continuously increasing levels of systemic OC after long-bone fractures over a 2-month period, which was felt to reflect bone remodeling.¹⁶³

In recent years, PINP has been introduced into clinical practice as a new tool for the measurement of bone turnover. An analytic kit for serum measurement is now commercially available (Roche Elecsys; Hoffman-LaRoche Inc, Penzberg, Germany), but it is not currently used with humans in the United States outside of research studies. However, since 2007, PINP has been licensed in Europe for diagnostic purposes, as well as for research, clinical studies, or trials.¹⁶⁴

As an indicator of type I collagen production, PINP can be useful in the assessment of skeletal regeneration in healthy and abnormal bone. Researchers have described a change in collagen production during fracture healing.^{165–169} In humans, PINP has shown different kinetics in long-bone fracture versus osteoporosis,¹⁶⁵ as shown in results from a bone turnover assessment in a prospective, longitudinal study of 85 elderly women with a trauma-related fracture (taken from a sample of 1044 elderly women in the Malmö OPRA study¹⁶⁵). Furthermore, these authors showed that most BTMs (OC and PINP) were significantly elevated during repair for several months after fracture and remained at elevated levels for up to 1 year. A significant increase was observed at 4 months relative to preinjury measurements or to patients without fractures. No significant increase of urine OC was noticeable. The most predominant fracture type was at the hip. The increases in PINP and OC levels were similar (51% vs. 67%, respectively). A delayed peak and prolonged period of elevation in OC was observed. A time-dependent association for serum PINP could not be shown in this diverse group.¹⁶⁵ However, PINP levels were characterized as an index of collagen synthesis and a marker of the early stages of bone formation.¹⁶⁵

In a prospective evaluation of biochemical bone marker response to distal forearm fracture, researchers noted a maximum increase of bone-specific ALP between 2 to 4 weeks; a slower, but significantly elevated, increase in OC compared with ALP; and a greater increase in PINP compared with other formation markers after fracture.¹⁶⁷ PINP was significantly elevated at 3 days and reached a maximal increase of 55% at 6 weeks, relative to a maximum increase of OC at 26 weeks. In a recent experimental study on the dose-dependent effect of PTH in an osteoporotic femoral cortical defect model, a peak serum concentration of PINP at 21 days was observed.¹⁶⁹ Interestingly, collagen type I mRNA levels peaked at day 7 with a significant 54-fold increase, followed by a sharp reduction at day 21 to a still-significant 10-fold elevation, whereas OC peaked at day 7 with a 9-fold increase and remained elevated until day 21.

Veitch et al. analyzed BTMs in patients with tibial shaft fractures who received cast immobilization or intramedullary nailing.¹⁷⁰ All fractures healed. A persistent increase in PINP occurred throughout a 24-week period, with a peak in serum concentrations at 12 weeks. Chevrel et al. showed a significant difference in serum concentration of PINP between healthy men and women after fracture of various bones.¹⁷¹ In another study of bone

turnover after distal forearm fractures in postmenopausal women,¹⁶⁶ Ingle et al. showed that PINP concentrations peaked 6 weeks after trauma, with elevated serum levels persisting at 1 year. The authors concluded that PINP levels might be associated with callus formation. No comment was made on the correlation of PINP levels with fracture healing. Analyses of femoral metaphyseal defects revealed increased mRNA levels of markers of bone formation (pro α 1(I) and OC) with maximum concentration for type I collagen at 7 days and OC at 21 days in a mouse cortical bone defect model.¹⁵⁵

Other related studies have linked changes in PINP levels to healing. In a study on collagen turnover of the Achilles tendon in young adult patients with concomitant ankle fracture, a significant increase in serum PINP concentrations with a peak at 6 weeks during immobilization was shown.¹⁶⁸ In addition, an increase in local PINP concentrations within the tendon was shown. After the immobilization period, PINP serum concentration returned to baseline, which differed from the findings of Ingle et al.¹⁶⁶

Other studies have shown that the degree of physical activity (immobilization, exercise, endurance running) is directly associated with an effect on BTMs.^{98,172} Cramer et al. showed that high-intensity eccentric exercise is associated with an increase in procollagen processing, including a significant increase in PINP in skeletal muscle.¹⁷³

Forthcoming preliminary data from our experimental study on 12-week-old male and female C57/BL6 mice, in which a closed mid-diaphyseal femoral fracture was induced after retrograde insertion of a 25 gauge intramedullary pin, revealed that serum PINP levels before fracture and at days 7 and 23 postfracture showed no significant differences compared with a control group without fracture. (Burgers TA, Coulibaly MO, Williams BO, Sietsema DL, Jones CB, and Mason J. PINP expression during femoral fracture healing in mice. [submitted]). Significant differences in PINP expression between the groups were noted at days 11 and 17 postfracture ($P < .05$) and corresponded to radiographic callus formation. In the fracture group, on days 11 and 17, the PINP expression was significantly different ($P < .05$) from the prefracture PINP levels. The animals that underwent femoral fracture showed higher concentrations of PINP ($P < .05$). We concluded from these preliminary data that PINP has potential as a blood serum marker for proper healing when applied at the correct time interval. Higher concentrations of PINP in mice later after fracture reflected collagen type I synthesis and callus formation.

VIII. BONE FORMATION MARKERS AS SEROLOGICAL MARKERS OF NONUNION

An early study indicated that in patients with established nonunion (time from index injury ranged from 15 months to 8 years), measurement of serum ALP or OC revealed no different values relative to healthy controls.¹⁷⁴ However, correlations of delayed fracture healing with reduced serum OC concentration have been reported.⁹³ Observations have been made for OC and ALP that show a positive correlation at injury and 6 weeks after fracture, but no correlation at 12 weeks.¹⁵⁸ Using an infected femoral diaphyseal defect model, Southwood et al. demonstrated that serum OC concentration was not useful for predicting fracture infection status compared with serum bone-specific ALP.¹⁷⁵ Rabbits with infected fractures had significantly lower serum ALP concentrations at 4 weeks relative to those with non-infected fractures. However, no difference in serum ALP concentration was found between united and nonunited fracture defects. Serum ALP concentration was not found to be a useful tool to predict fracture healing, although a significant association was found between the radiographic external callus grade and serum ALP concentration.

In a prospective evaluation of patients having tibial fractures with delayed healing, Joerring et al. demonstrated an early increase in serum concentrations of PICP, ICTP, and PIIINP. The authors concluded that delayed fracture healing was caused by an early increase in turnover of collagen type I and III.¹⁶² Kurdy et al. reported a persistent decline in PICP levels from 10 weeks onward in patients with nonhealing tibial fractures.¹⁵⁹ A progressive increase in ALP levels, although not as pronounced, was found in nonhealing fractures, with no difference relative to healing fractures.

Interestingly, PINP, although characterized as an index of collagen synthesis and marker of early stages of bone healing,¹⁶⁵ has not yet been evaluated as an indicator of failed fracture healing.

IX. SUMMARY

Currently, there is no reliable way to predict which patients will suffer from nonunion. The development of a method to do so would allow for early intervention to reduce the complications of nonunion and the associated morbidity.^{24,25} Furthermore, such early intervention could shorten the recovery time significantly, resulting in a significant impact on health care system costs and society.^{26–28} However, the early intervention must be warranted by a reliable indication of nonunion to prevent unnecessary intervention and possible complications. Despite the many advances in treatment techniques, implant design, and appliances, diagnostic measures and clinical knowledge of fracture healing remain unpredictable in many cases. To obviate scar formation during fracture healing, bone reverts to the complex temporal and spatial developmental processes of embryological growth—a nearly unique characteristic. Thus, fracture healing is multifactorially influenced, and science thus far has not solved the problem of predicting disturbed fracture healing.

Fracture healing is dependent on a balanced transition of extracellular matrix components during the key processes of chondrogenesis and ossification, in which collagen type I plays a major role. The essential phases for bone formation are inflammation and reparation; thus, 2 weeks after fracture, healing is reflected by the dynamic pattern of expression of procollagen type I within the healing bone (callus). The biochemical characteristics of collagen type I synthesis in bone, with the cleavage of its terminal ends and release of those ends into the interstitial space, make this molecule accessible to serum analysis.

Advantages of serological analysis of human blood during fracture healing include ease of use, less invasion, and less temporal variation, which are not generally possible via histological examination of biopsies. Problems with serological analysis of collagen type I as a reflection of the histology of fracture healing include a lack of differentiation between local, regional, or systemic responses to the fracture. Collagen type I is found in other tissues such as liver, skin, and bowel, and it has been shown that the rate of turnover for other tissues is comparable to that of bone.¹⁷⁶ However, the amount of collagen type I in other tissues is low relative to that of bone; therefore, changes in the rate of bone collagen synthesis should correlate well with and reflect changes in the rate of bone formation during healing.

For these reasons, it remains important to critically evaluate the value of bone formation markers as indications of fracture healing. Bone-specific ALP, despite being the bone formation marker available for the longest period and having biochemically favorable characteristics (e.g., analytic kits with high specificity, minimal diurnal variation), has relatively small changes in serum level during fracture healing and has not been found indicative in some studies. Although OC is the most abundant noncollagen protein in bone matrix, its biochemical characteristics that reflect both bone regeneration and resorption make it an unreliable tool, which is seen in the current data. OC is also similar to ALP in

that it results in relatively small changes in serum levels during healing. PICP is the collagen type I extension that has been investigated for the longest period and that has been related to bone remodeling and the status of bone healing. However, PICP level changes are also generally smaller than changes in PINP.

So far, PINP has been characterized as age- and gender-dependent, and shows minimal diurnal variation. Changes in serum concentrations in PINP have been found to be high during fracture. In this regard, PINP is superior in reflecting bone formation processes when compared with ALP, PICP, and OC. PINP can also be characterized as an index of collagen synthesis, a marker of the early stages of bone formation, and a marker of callus formation. Consequently, it is our opinion that PINP is the best candidate for use as a serological marker of bone healing. However, the temporal changes of PINP during disturbed fracture healing are unknown and require further elucidation.

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REFERENCES

1. Nawar EW, Niska RW, Xu J. National Hospital Ambulatory Medical Care Survey: 2005 emergency department summary. *Adv Data*. 2007; (386):1–32. [PubMed: 17703794]
2. Donaldson LJ, Cook A, Thomson RG. Incidence of fractures in a geographically defined population. *J Epidemiol Community Health*. 1990; 44(3):241–245. [PubMed: 2273364]
3. Banks E, Reeves GK, Beral V, Balkwill A, Liu B, Roddam A. for the Million Women Study C. Hip fracture incidence in relation to age, menopausal status, and age at menopause: prospective analysis. *PLoS Med*. 2009; 6(11):e1000181. [PubMed: 19901981]
4. Court-Brown CM, Caesar B. Epidemiology of adult fractures: A review. *Injury*. 2006; 37(8):691–697. [PubMed: 16814787]
5. 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):873–884. [PubMed: 12616527]
6. Nork, SE. Fractures of the Shaft of the Femur. In: Bucholz, RW.; Heckman, JD.; Court-Brown, CM., editors. *Rockwood and Green's Fractures in Adults*. 6th ed.. Philadelphia (PA): Lippincott Williams & Wilkins; 2006. p. 1845-1914.
7. US Food and Drug Administration. Premarket Approval P900009/Supplement S006. Exogen 2000®: summary of safety and effectiveness. Low-intensity pulsed ultrasound device for the noninvasive treatment of nonunions. Available from: http://www.accessdata.fda.gov/cdrh_docs/pdf/P900009S006b.pdf (published 2000 Feb 22; cited 2010 Oct 10)
8. Court-Brown CM, McQueen MM. Nonunions of the proximal humerus: their prevalence and functional outcome. *J Trauma*. 2008; 64(6):1517–1521. [PubMed: 18545116]
9. Parker MJ, Raghavan R, Gurusamy K. Incidence of fracture-healing complications after femoral neck fractures. *Clin Orthop Relat Res*. 2007; 458:175–179. [PubMed: 17224836]
10. Giannoudis PV, MacDonald DA, Matthews SJ, Smith RM, Furlong AJ, De Boer P. Nonunion of the femoral diaphysis. The influence of reaming and non-steroidal anti-inflammatory drugs. *J Bone Joint Surg Br*. 2000; 82(5):655–658. [PubMed: 10963160]
11. Society COT. Nonunion following intramedullary nailing of the femur with and without reaming. Results of a multicenter randomized clinical trial. *J Bone Joint Surg Am*. 2003; 85-A(11):2093–2096. [PubMed: 14630836]
12. Phieffer LS, Goulet JA. Delayed unions of the tibia. *Instr Course Lect*. 2006; 55:389–401. [PubMed: 16958474]

13. Taitsman LA, Lynch JR, Agel J, Barei DP, Nork SE. Risk factors for femoral nonunion after femoral shaft fracture. *J Trauma*. 2009; 67(6):1389–1392. [PubMed: 19704386]
14. Ly TV, Swiontkowski MF. Treatment of femoral neck fractures in young adults. *J Bone Joint Surg Am*. 2008; 90(10):2254–2266. [PubMed: 18829925]
15. Yokoyama K, Itoman M, Uchino M, Fukushima K, Nitta H, Kojima Y. Immediate versus delayed intramedullary nailing for open fractures of the tibial shaft: A multivariate analysis of factors affecting deep infection and fracture healing. *Indian J Orthop*. 2008; 42(4):410–419. [PubMed: 19753228]
16. Loder RT. The influence of diabetes mellitus on the healing of closed fractures. *Clin Orthop Relat Res*. 1988; (232):210–216. [PubMed: 3289812]
17. Foulk DA, Szabo RM. Diaphyseal humerus fractures: natural history and occurrence of nonunion. *Orthopedics*. 1995; 18(4):333–335. [PubMed: 7603916]
18. Lindsey RW, Blair SR. Closed tibial-shaft fractures: which ones benefit from surgical treatment? *J Am Acad Orthop Surg*. 1996; 4(1):35–43. [PubMed: 10795037]
19. Kyro A, Usenius JP, Aarnio M, Kunnamo I, Avikainen V. Are smokers a risk group for delayed healing of tibial shaft fractures? *Ann Chir Gynaecol*. 1993; 82(4):254–262. [PubMed: 8122874]
20. Adams CI, Keating JF, Court-Brown CM. Cigarette smoking and open tibial fractures. *Injury*. 2001; 32(1):61–65. [PubMed: 11164405]
21. Ueng SW, Chao EK, Lee SS, Shih CH. Augmentative plate fixation for the management of femoral nonunion after intramedullary nailing. *J Trauma*. 1997; 43(4):640–644. [PubMed: 9356061]
22. Whelan DB, Bhandari M, Stephen D, Kreder H, McKee MD, Zdero R, Schemitsch EH. Development of the radiographic union score for tibial fractures for the assessment of tibial fracture healing after intramedullary fixation. *J Trauma*. 2010; 68(3):629–632. [PubMed: 19996801]
23. Kuhlman JE, Fishman EK, Magid D, Scott WW Jr, Brooker AF, Siegelman SS. Fracture nonunion: CT assessment with multiplanar reconstruction. *Radiology*. 1988; 167(2):483–488. [PubMed: 3357959]
24. Webb LX, Winquist RA, Hansen ST. Intramedullary nailing and reaming for delayed union or nonunion of the femoral shaft. A report of 105 consecutive cases. *Clin Orthop Relat Res*. 1986; (212):133–141. [PubMed: 3769279]
25. Wu CC. The effect of dynamization on slowing the healing of femur shaft fractures after interlocking nailing. *J Trauma*. 1997; 43(2):263–267. [PubMed: 9291371]
26. Weresh MJ, Hakanson R, Stover MD, Sims SH, Kellam JF, Bosse MJ. Failure of exchange reamed intramedullary nails for ununited femoral shaft fractures. *J Orthop Trauma*. 2000; 14(5):335–338. [PubMed: 10926240]
27. Patil S, Montgomery R. Management of complex tibial and femoral nonunion using the Ilizarov technique, and its cost implications. *J Bone Joint Surg Br*. 2006; 88(7):928–932. [PubMed: 16798998]
28. Dahabreh Z, Calori GM, Kanakaris NK, Nikolaou VS, Giannoudis PV. A cost analysis of treatment of tibial fracture nonunion by bone grafting or bone morphogenetic protein-7. *Int Orthop*. 2009; 33(5):1407–1414. [PubMed: 19052743]
29. Ramachandran GN, Kartha G. Structure of collagen. *Nature*. 1954; 174(4423):269–270. [PubMed: 13185286]
30. Weiner S, Traub W, Wagner HD. Lamellar bone: structure-function relations. *J Struct Biol*. 1999; 126(3):241–255. [PubMed: 10475685]
31. Traub W, Piez KA. The chemistry and structure of collagen. *Adv Protein Chem*. 1971; 25:243–352. [PubMed: 4111984]
32. Helseth DL Jr, Veis A. Collagen self-assembly in vitro. Differentiating specific telopeptide-dependent interactions using selective enzyme modification and the addition of free amino telopeptide. *J Biol Chem*. 1981; 256(14):7118–7128. [PubMed: 7251588]
33. Bornstein P, Ehrlich HP, Wyke AW. Procollagen: conversion of the precursor to collagen by a neutral protease. *Science*. 1972; 175(21):544–546. [PubMed: 5008597]

34. Bornstein P, Von der Mark K, Wyke AW, Ehrlich HP, Monson JM. Characterization of the pro- $\alpha 1$ chain of procollagen. *J Biol Chem.* 1972; 247(9):2808–2813. [PubMed: 4337102]
35. Fessler LI, Morris NP, Fessler JH. Procollagen: biological scission of amino and carboxyl extension peptides. *Proc Natl Acad Sci U S A.* 1975; 72(12):4905–4909. [PubMed: 1061079]
36. Miyahara M, Hayashi K, Berger J, Tanzawa K, Njieha FK, Trelstad RL, Prockop DJ. Formation of collagen fibrils by enzymic cleavage of precursors of type I collagen in vitro. *J Biol Chem.* 1984; 259(15):9891–9898. [PubMed: 6430905]
37. Prockop DJ, Kivirikko KI, Tuderman L, Guzman NA. The biosynthesis of collagen and its disorders (second of two parts). *N Engl J Med.* 1979; 301(2):77–85. [PubMed: 36559]
38. Prockop DJ, Kivirikko KI, Tuderman L, Guzman NA. The biosynthesis of collagen and its disorders (first of two parts). *N Engl J Med.* 1979; 301(1):13–23. [PubMed: 449904]
39. Hojima Y, Morgelin MM, Engel J, Boutillon MM, van der Rest M, McKenzie J, Chen GC, Rafi N, Romanic AM, Prockop DJ. Characterization of type I procollagen N-proteinase from fetal bovine tendon and skin. Purification of the 500-kilodalton form of the enzyme from bovine tendon. *J Biol Chem.* 1994; 269(15):11381–11390. [PubMed: 8157670]
40. Prockop DJ, Sieron AL, Li SW. Procollagen N-proteinase and procollagen C-proteinase. Two unusual metalloproteinases that are essential for procollagen processing probably have important roles in development and cell signaling. *Matrix Biol.* 1998; 16(7):399–408. [PubMed: 9524360]
41. Weiner S, Traub W. Bone structure: from angstroms to microns. *FASEB J.* 1992; 6(3):879–885. [PubMed: 1740237]
42. Hodge AJ. Molecular models illustrating the possible distributions of 'holes' in simple systematically staggered arrays of type I collagen molecules in native-type fibrils. *Connect Tissue Res.* 1989; 21(1–4):137–147. [PubMed: 2605937]
43. Otsubo K, Katz EP, Mechanic GL, Yamauchi M. Cross-linking connectivity in bone collagen fibrils: the COOH-terminal locus of free aldehyde. *Biochemistry.* 1992; 31(2):396–402. [PubMed: 1731897]
44. Robins SP. Cross-linking of collagen. Isolation, structural characterization and glycosylation of pyridinoline. *Biochem J.* 1983; 215(1):167–173. [PubMed: 6626172]
45. Gunja-Smith Z, Boucek RJ. Collagen cross-linking compounds in human urine. *Biochem J.* 1981; 197(3):759–762. [PubMed: 7325985]
46. Ge G, Seo NS, Liang X, Hopkins DR, Hook M, Greenspan DS. Bone morphogenetic protein-1/tollid-related metalloproteinases process osteoglycin and enhance its ability to regulate collagen fibrillogenesis. *J Biol Chem.* 2004; 279(40):41626–41633. [PubMed: 15292192]
47. Veis A, Anesey J, Yuan L, Levy SJ. Evidence for an amino-terminal extension in high-molecular-weight collagens from mature bovine skin. *Proc Natl Acad Sci U S A.* 1973; 70(5):1464–1467. [PubMed: 4576019]
48. Bornstein P, Walsh V, Tullis J, Stainbrook E, Bateman JF, Hormuzdi SG. The globular domain of the pro $\alpha 1$ (I) N-propeptide is not required for secretion, processing by procollagen N-proteinase, or fibrillogenesis of type I collagen in mice. *J Biol Chem.* 2002; 277(4):2605–2613. [PubMed: 11705995]
49. Fisher LW, Robey PG, Tuross N, Otsuka AS, Tepen DA, Esch FS, Shimasaki S, Termine JD. The Mr 24,000 phosphoprotein from developing bone is the NH₂-terminal propeptide of the $\alpha 1$ chain of type I collagen. *J Biol Chem.* 1987; 262(28):13457–13463. [PubMed: 3654622]
50. Cabral WA, Makareeva E, Colige A, Letocha AD, Ty JM, Yeowell HN, Pals G, Leikin S, Marini JC. Mutations near amino end of $\alpha 1$ (I) collagen cause combined osteogenesis imperfecta/Ehlers-Danlos syndrome by interference with N-propeptide processing. *J Biol Chem.* 2005; 280(19):19259–19269. [PubMed: 15728585]
51. Pope FM, Burrows NP. Ehlers-Danlos syndrome has varied molecular mechanisms. *J Med Genet.* 1997; 34(5):400–410. [PubMed: 9152838]
52. Rauch F, Lalic L, Roughley P, Glorieux FH. Relationship between genotype and skeletal phenotype in children and adolescents with osteogenesis imperfecta. *J Bone Miner Res.* 2010; 25(6):1367–1374. [PubMed: 19929435]

53. Meyer RA Jr, Desai BR, Heiner DE, Fiechtel J, Porter S, Meyer MH. Young, adult, and old rats have similar changes in mRNA expression of many skeletal genes after fracture despite delayed healing with age. *J Orthop Res.* 2006; 24(10):1933–1944. [PubMed: 16894589]
54. Ferguson CM, Miclau T, Hu D, Alpern E, Helms JA. Common molecular pathways in skeletal morphogenesis and repair. *Ann N Y Acad Sci.* 1998; 857:33–42. [PubMed: 9917830]
55. Kaplan, FS.; Hayes, WC.; Keaveny, TM.; Boskey, A.; Einhorn, TA.; Iannotti, JP. Form and function of bone. In: Simon, SS., editor. *Orthopaedic basic science*. Rosemont (IL): American Academy of Orthopaedic Surgeons; 1994. p. 127-184.
56. Linkhart SG, Linkhart TA, Taylor AK, Wergedal JE, Bettica P, Baylink DJ. Synthetic peptide-based immunoassay for amino-terminal propeptide of type I procollagen: application for evaluation of bone formation. *Clin Chem.* 1993; 39(11 Pt 1):2254–2258. [PubMed: 8222218]
57. Buckwalter, JA.; Einhorn, TA.; Marsh, JL. Bone and joint healing. In: Bucholz, RW.; Heckman, JD.; Court-Brown, CM., editors. *Rockwood and Green's fractures in adults*. 6th ed.. Philadelphia (PA): Lippincott Williams & Wilkins; 2006. p. 297-312.
58. Reddi AH, Gay R, Gay S, Miller EJ. Transitions in collagen types during matrix-induced cartilage, bone, and bone marrow formation. *Proc Natl Acad Sci U S A.* 1977; 74(12):5589–5592. [PubMed: 271986]
59. Page M, Hogg J, Ashhurst DE. The effects of mechanical stability on the macromolecules of the connective tissue matrices produced during fracture healing. I. The collagens. *Histochem J.* 1986; 18(5):251–265. [PubMed: 3528065]
60. Gerstenfeld LC, Alkhiary YM, Krall EA, Nicholls FH, Stapleton SN, Fitch JL, Bauer M, Kayal R, Graves DT, Jepsen KJ, Einhorn TA. Three-dimensional reconstruction of fracture callus morphogenesis. *J Histochem Cytochem.* 2006; 54(11):1215–1228. [PubMed: 16864894]
61. McKibbin B. The biology of fracture healing in long bones. *J Bone Joint Surg Br.* 1978; 60-B(2): 150–162. [PubMed: 350882]
62. Reddi AH, Anderson WA. Collagenous bone matrix-induced endochondral ossification hemopoiesis. *J Cell Biol.* 1976; 69(3):557–572. [PubMed: 1270511]
63. Lane JM, Suda M, von der Mark K, Timpl R. Immunofluorescent localization of structural collagen types in endochondral fracture repair. *J Orthop Res.* 1986; 4(3):318–329. [PubMed: 3525787]
64. Sandberg M, Aro H, Multimaki P, Aho H, Vuorio E. In situ localization of collagen production by chondrocytes and osteoblasts in fracture callus. *J Bone Joint Surg Am.* 1989; 71(1):69–77. [PubMed: 2643609]
65. Multimaki P, Aro H, Vuorio E. Differential expression of fibrillar collagen genes during callus formation. *Biochem Biophys Res Commun.* 1987; 142(2):536–541. [PubMed: 3814147]
66. Chiba S, Okada K, Lee K, Segre GV, Neer RM. Molecular analysis of defect healing in rat diaphyseal bone. *J Vet Med Sci.* 2001; 63(6):603–608. [PubMed: 11459005]
67. Yamagiwa H, Tokunaga K, Hayami T, Hatano H, Uchida M, Endo N, Takahashi HE. Expression of metalloproteinase-13 (Collagenase-3) is induced during fracture healing in mice. *Bone.* 1999; 25(2):197–203. [PubMed: 10456385]
68. Kon T, Cho TJ, Aizawa T, Yamazaki M, Nooh N, Graves D, Gerstenfeld LC, Einhorn TA. Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. *J Bone Miner Res.* 2001; 16(6): 1004–1014. [PubMed: 11393777]
69. Hiltunen A, Aro HT, Vuorio E. Regulation of extracellular matrix genes during fracture healing in mice. *Clin Orthop Relat Res.* 1993; (297):23–27. [PubMed: 8242936]
70. Riikonen T, Westermarck J, Koivisto L, Broberg A, Kahari VM, Heino J. Integrin alpha 2 beta 1 is a positive regulator of collagenase (MMP-1) and collagen alpha 1(I) gene expression. *J Biol Chem.* 1995; 270(22):13548–13552. [PubMed: 7768957]
71. Riikonen T, Koivisto L, Vihinen P, Heino J. Transforming growth factor-beta regulates collagen gel contraction by increasing alpha 2 beta 1 integrin expression in osteogenic cells. *J Biol Chem.* 1995; 270(1):376–382. [PubMed: 7529233]

72. Ekholm E, Hankenson KD, Uusitalo H, Hiltunen A, Gardner H, Heino J, Penttinen R. Diminished callus size and cartilage synthesis in alpha 1 beta 1 integrin-deficient mice during bone fracture healing. *Am J Pathol.* 2002; 160(5):1779–1785. [PubMed: 12000729]
73. Pei D. Identification and characterization of the fifth membrane-type matrix metalloproteinase MT5-MMP. *J Biol Chem.* 1999; 274(13):8925–8932. [PubMed: 10085137]
74. Werb Z, Chin JR. Extracellular matrix remodeling during morphogenesis. *Ann N Y Acad Sci.* 1998; 857:110–118. [PubMed: 9917836]
75. Mauviel A. Cytokine regulation of metalloproteinase gene expression. *J Cell Biochem.* 1993; 53(4):288–295. [PubMed: 8300745]
76. Marsh D. Concepts of fracture union, delayed union, and nonunion. *Clin Orthop Relat Res.* 1998; (355 Suppl):S22–S30. [PubMed: 9917623]
77. Weber, BG.; Cech, O. Pseudarthrosen: pathophysiologie, biomechanik, therapie, ergebnisse. Bern, Switzerland: Verlag Hans Huber; 1973.
78. Segmuller G, Cech O, Bekier A. [Osteogenic activity in the area of pseudarthrosis of the long tubular bones]. *Z Orthop Ihre Grenzgeb.* 1969; 106(3):599–609. [PubMed: 4242695]
79. Reed AA, Joyner CJ, Isefuku S, Brownlow HC, Simpson AH. Vascularity in a new model of atrophic nonunion. *J Bone Joint Surg Br.* 2003; 85(4):604–610. [PubMed: 12793572]
80. Hietaniemi K, Lehto MU, Paavolainen P. Major fibrillar collagens and fibronectin in an experimental nonunion: an immunohistochemical study. *Acta Orthop Scand.* 1998; 69(5):545–549. [PubMed: 9855241]
81. Brick KE, Chen X, Lohr J, Schmidt AH, Kidder LS, Lew WD. rhBMP-2 modulation of gene expression in infected segmental bone defects. *Clin Orthop Relat Res.* 2009; 467(12):3096–3103. [PubMed: 19018606]
82. Lawton DM, Andrew JG, Marsh DR, Hoyland JA, Freemont AJ. Expression of the gene encoding the matrix gla protein by mature osteoblasts in human fracture non-unions. *Mol Pathol.* 1999; 52(2):92–96. [PubMed: 10474688]
83. Lawton DM, Andrew JG, Marsh DR, Hoyland JA, Freemont AJ. Mature osteoblasts in human non-union fractures express collagen type III. *Mol Pathol.* 1997; 50(4):194–197. [PubMed: 9350302]
84. Paralkar VM, Weeks BS, Yu YM, Kleinman HK, Reddi AH. Recombinant human bone morphogenetic protein 2B stimulates PC12 cell differentiation: potentiation and binding to type IV collagen. *J Cell Biol.* 1992; 119(6):1721–1728. [PubMed: 1469059]
85. Herrmann M, Klitscher D, Georg T, Frank J, Marzi I, Herrmann W. Different kinetics of bone markers in normal and delayed fracture healing of long bones. *Clin Chem.* 2002; 48(12):2263–2266. [PubMed: 12446490]
86. Henle P, Zimmermann G, Weiss S. Matrix metalloproteinases and failed fracture healing. *Bone.* 2005; 37(6):791–798. [PubMed: 16199217]
87. Buttaro MA, Gonzalez Della Valle AM, Pineiro L, Mocetti E, Morandi AA, Piccaluga F. Incorporation of vancomycin-supplemented bone incorporation of vancomycin-supplemented bone allografts: radiographical, histopathological and immunohistochemical study in pigs. *Acta Orthop Scand.* 2003; 74(5):505–513. [PubMed: 14620969]
88. Waters RV, Gamradt SC, Asnis P, Vickery BH, Avnur Z, Hill E, Bostrom M. Systemic corticosteroids inhibit bone healing in a rabbit ulnar osteotomy model. *Acta Orthop Scand.* 2000; 71(3):316–321. [PubMed: 10919307]
89. Iu MF, Kaji H, Sowa H, Naito J, Sugimoto T, Chihara K. Dexamethasone suppresses Smad3 pathway in osteoblastic cells. *J Endocrinol.* 2005; 185(1):131–138. [PubMed: 15817834]
90. Nagel T, Kelly DJ. Mechano-regulation of mesenchymal stem cell differentiation and collagen organisation during skeletal tissue repair. *Biomech Model Mechanobiol.* 2010; 9(3):359–372. [PubMed: 20039092]
91. Rothem DE, Rothem L, Soudry M, Dahan A, Eliakim R. Nicotine modulates bone metabolism-associated gene expression in osteoblast cells. *J Bone Miner Metab.* 2009; 27(5):555–561. [PubMed: 19436947]
92. Einhorn TA, Gundberg CM, Devlin VJ, Warman J. Fracture healing and osteocalcin metabolism in vitamin K deficiency. *Clin Orthop Relat Res.* 1988; (237):219–225. [PubMed: 3263905]

93. Oni OO, Mahabir JP, Iqbal SJ, Gregg PJ. Serum osteocalcin and total alkaline phosphatase levels as prognostic indicators in tibial shaft fractures. *Injury*. 1989; 20(1):37–38. [PubMed: 2592063]
94. Delmas PD, Stenner D, Wahner HW, Mann KG, Riggs BL. Increase in serum bone gamma-carboxyglutamic acid protein with aging in women. Implications for the mechanism of age-related bone loss. *J Clin Invest*. 1983; 71(5):1316–1321. [PubMed: 6304145]
95. Jones ED. Effects of local alkaline phosphatase in experimentally produced fracture in the rabbit. *Am J Surg*. 1951; 81(4):417–420. [PubMed: 14819493]
96. Sieber E. [The significance of alkaline serum phosphatase analysis in the course of bone fracture healing.]. *Zentralbl Chir*. 1953; 78(37):1583–1590. [PubMed: 13123331]
97. Delmas PD, Eastell R, Garnero P, Seibel MJ, Stepan J. The use of biochemical markers of bone turnover in osteoporosis. Committee of Scientific Advisors of the International Osteoporosis Foundation. *Osteoporos Int*. 2000; 11 Suppl 6:S2–S17. [PubMed: 11193237]
98. Adami S, Gatti D, Viapiana O, Fiore CE, Nuti R, Luisetto G, Ponte M, Rossini M. Physical activity and bone turnover markers: a cross-sectional and a longitudinal study. *Calcif Tissue Int*. 2008; 83(6):388–392. [PubMed: 18949504]
99. Seebeck P, Bail HJ, Exner C, Schell H, Michel R, Amthauer H, Bragulla H, Duda GN. Do serological tissue turnover markers represent callus formation during fracture healing? *Bone*. 2005; 37(5):669–677. [PubMed: 16126014]
100. Gothlin G, Ericsson JL. Fine structural localization of alkaline phosphatase in the fracture callus of the rat. *Histochemie*. 1973; 36(3):225–236. [PubMed: 4757993]
101. Felix R, Herrmann W, Fleisch H. Stimulation of precipitation of calcium phosphate by matrix vesicles. *Biochem J*. 1978; 170(3):681–691. [PubMed: 646808]
102. Anderson HC, Stechschulte DJ Jr, Collins DE, Jacobs DH, Morris DC, Hsu HH, Redford PA, Zeiger S. Matrix vesicle biogenesis in vitro by rachitic and normal rat chondrocytes. *Am J Pathol*. 1990; 136(2):391–398. [PubMed: 2305834]
103. Moss DW. Alkaline phosphatase isoenzymes. *Clin Chem*. 1982; 28(10):2007–2016. [PubMed: 6751596]
104. Hill CS, Wolfert RL. The preparation of monoclonal antibodies which react preferentially with human bone alkaline phosphatase and not liver alkaline phosphatase. *Clin Chim Acta*. 1990; 186(2):315–320. [PubMed: 2178811]
105. Posen S, Grunstein HS. Turnover rate of skeletal alkaline phosphatase in humans. *Clin Chem*. 1982; 28(1):153–154. [PubMed: 7055900]
106. Denney JD, Sherrard DJ, Nelp WB, Chesnut CH3rd, Baylink DJ. Total body calcium and long-term calcium balance in chronic renal disease. *J Lab Clin Med*. 1973; 82(2):226–240. [PubMed: 4721378]
107. Manolagas SC, Spiess YH, Burton DW, Deftos LJ. Mechanism of action of 1,25-dihydroxyvitamin D3-induced stimulation of alkaline phosphatase in cultured osteoblast-like cells. *Mol Cell Endocrinol*. 1983; 33(1):27–36. [PubMed: 6357897]
108. Manolagas SC, Burton DW, Deftos LJ. 1,25-Dihydroxyvitamin D3 stimulates the alkaline phosphatase activity of osteoblast-like cells. *J Biol Chem*. 1981; 256(14):7115–7117. [PubMed: 6941964]
109. Duda RJ Jr, Kumar R, Nelson KI, Zinsmeister AR, Mann KG, Riggs BL. 1,25-Dihydroxyvitamin D stimulation test for osteoblast function in normal and osteoporotic postmenopausal women. *J Clin Invest*. 1987; 79(4):1249–1253. [PubMed: 3494046]
110. Hammett-Stabler CA. The use of biochemical markers in osteoporosis. *Clin Lab Med*. 2004; 24(1):175–197. [PubMed: 15157562]
111. Han B, Copeland M, Geiser AG, Hale LV, Harvey A, Ma YL, Powers CS, Sato M, You J, Hale JE. Development of a highly sensitive, high-throughput, mass spectrometry-based assay for rat procollagen type-I N-terminal propeptide (PINP) to measure bone formation activity. *J Proteome Res*. 2007; 6(11):4218–4229. [PubMed: 17924680]
112. Melkko J, Kauppila S, Niemi S, Risteli L, Haukipuro K, Jukkola A, Risteli J. Immunoassay for intact amino-terminal propeptide of human type I procollagen. *Clin Chem*. 1996; 42(6 Pt 1):947–954. [PubMed: 8665688]

113. Fleischmajer R, Perlish JS, Burgeson RE, Shaikh-Bahai F, Timpl R. Type I and type III collagen interactions during fibrillogenesis. *Ann N Y Acad Sci.* 1990; 580:161–175. [PubMed: 2186689]
114. Melkko J, Hellevik T, Risteli L, Risteli J, Smedsrod B. Clearance of NH2-terminal propeptides of types I and III procollagen is a physiological function of the scavenger receptor in liver endothelial cells. *J Exp Med.* 1994; 179(2):405–412. [PubMed: 8294857]
115. Hale LV, Galvin RJ, Risteli J, Ma YL, Harvey AK, Yang X, Cain RL, Zeng Q, Frolik CA, Sato M, Schmidt AL, Geiser AG. PINP: a serum biomarker of bone formation in the rat. *Bone.* 2007; 40(4):1103–1109. [PubMed: 17258520]
116. Harrison CA, Gossiel F, Bullock AJ, Sun T, Blumsohn A, Mac Neil S. Investigation of keratinocyte regulation of collagen I synthesis by dermal fibroblasts in a simple in vitro model. *Br J Dermatol.* 2006; 154(3):401–410. [PubMed: 16445767]
117. Burnett-Bowie SA, Saag K, Sebba A, de Papp AE, Chen E, Rosenberg E, Greenspan SL. Prediction of changes in bone mineral density in postmenopausal women treated with once-weekly bisphosphonates. *J Clin Endocrinol Metab.* 2009; 94(4):1097–1103. [PubMed: 19141590]
118. Chen P, Satterwhite JH, Licata AA, Lewiecki EM, Sipos AA, Misurski DM, Wagman RB. Early changes in biochemical markers of bone formation predict BMD response to teriparatide in postmenopausal women with osteoporosis. *J Bone Miner Res.* 2005; 20(6):962–970. [PubMed: 15883636]
119. Claudon A, Vergnaud P, Valverde C, Mayr A, Klause U, Garnero P. New automated multiplex assay for bone turnover markers in osteoporosis. *Clin Chem.* 2008; 54(9):1554–1563. [PubMed: 18676587]
120. Delmas PD, Licata AA, Reginster JY, Crans GG, Chen P, Misurski DA, Wagman RB, Mitlak BH. Fracture risk reduction during treatment with teriparatide is independent of pretreatment bone turnover. *Bone.* 2006; 39(2):237–243. [PubMed: 16563890]
121. Denk E, Hillegonds D, Hurrell RF, Vogel J, Fattinger K, Hauselmann HJ, Kraenzlin M, Walczyk T. Evaluation of 41calcium as a new approach to assess changes in bone metabolism: effect of a bisphosphonate intervention in postmenopausal women with low bone mass. *J Bone Miner Res.* 2007; 22(10):1518–1525. [PubMed: 17576167]
122. Gallagher JC, Rosen CJ, Chen P, Misurski DA, Marcus R. Response rate of bone mineral density to teriparatide in postmenopausal women with osteoporosis. *Bone.* 2006; 39(6):1268–1275. [PubMed: 16884968]
123. Komi J, Lankinen KS, DeGregorio M, Heikkinen J, Saarikoski S, Tuppurainen M, Halonen K, Lammintausta R, Vaananen K, Ylikorkala O, Erkkola R. Effects of ospemifene and raloxifene on biochemical markers of bone turnover in postmenopausal women. *J Bone Miner Metab.* 2006; 24(4):314–318. [PubMed: 16816926]
124. Nakatsuka K, Nishizawa Y, Miura M. [Bone and bone related biochemical examinations. Bone and collagen related metabolites. Measurement of PICP and PINP in the evaluation of pharmaceutical treatment efficacy for osteoporosis]. *Clin Calcium.* 2006; 16(6):977–985. [PubMed: 16751694]
125. Watts NB, Miller PD, Kohlmeier LA, Sebba A, Chen P, Wong M, Krohn K. Vertebral fracture risk is reduced in women who lose femoral neck BMD with teriparatide treatment. *J Bone Miner Res.* 2009; 24(6):1125–1131. [PubMed: 19113918]
126. McClung M, Recker R, Miller P, Fiske D, Minkoff J, Kriegman A, Zhou W, Adera M, Davis J. Intravenous zoledronic acid 5 mg in the treatment of postmenopausal women with low bone density previously treated with alendronate. *Bone.* 2007; 41(1):122–128. [PubMed: 17468062]
127. Miller PD, Schwartz EN, Chen P, Misurski DA, Kregg JH. Teriparatide in postmenopausal women with osteoporosis and mild or moderate renal impairment. *Osteoporos Int.* 2007; 18(1):59–68. [PubMed: 17013567]
128. Bauer DC, Garnero P, Hochberg MC, Santora A, Delmas P, Ewing SK, Black DM. Pretreatment levels of bone turnover and the antifracture efficacy of alendronate: the fracture intervention trial. *J Bone Miner Res.* 2006; 21(2):292–299. [PubMed: 16418785]
129. Deal C, Omizo M, Schwartz EN, Eriksen EF, Cantor P, Wang J, Glass EV, Myers SL, Kregg JH. Combination teriparatide and raloxifene therapy for postmenopausal osteoporosis: results from a

- 6-month double-blind placebo-controlled trial. *J Bone Miner Res.* 2005; 20(11):1905–1911. [PubMed: 16234962]
130. Rissanen JP, Suominen MI, Peng Z, Morko J, Rasi S, Risteli J, Halleen JM. Short-term changes in serum PINP predict long-term changes in trabecular bone in the rat ovariectomy model. *Calcif Tissue Int.* 2008; 82(2):155–161. [PubMed: 18219436]
 131. Delmas PD. Biochemical markers of bone turnover in Paget's disease of bone. *J Bone Miner Res.* 1999; 14 Suppl 2:66–69. [PubMed: 10510216]
 132. Alvarez L, Peris P, Guanabens N, Vidal S, Quinto L, Monegal A, Pons F, Ballesta AM, Munoz-Gomez J. Long-term biochemical response after bisphosphonate therapy in Paget's disease of bone. Proposed intervals for monitoring treatment. *Rheumatology (Oxford).* 2004; 43(7):869–874. [PubMed: 15054158]
 133. Alvarez L, Guanabens N, Peris P, Vidal S, Ros I, Monegal A, Bedini JL, Deulofeu R, Pons F, Munoz-Gomez J, Ballesta AM. Usefulness of biochemical markers of bone turnover in assessing response to the treatment of Paget's disease. *Bone.* 2001; 29(5):447–452. [PubMed: 11704497]
 134. Lein M, Miller K, Wirth M, Weissbach L, May C, Schmidt K, Haus U, Schrader M, Jung K. Bone turnover markers as predictive tools for skeletal complications in men with metastatic prostate cancer treated with zoledronic acid. *Prostate.* 2009; 69(6):624–632. [PubMed: 19143027]
 135. Body JJ, Lichinitser M, Tjulandin S, Garnero P, Bergstrom B. Oral ibandronate is as active as intravenous zoledronic acid for reducing bone turnover markers in women with breast cancer and bone metastases. *Ann Oncol.* 2007; 18(7):1165–1171. [PubMed: 17442659]
 136. Goss PE, Hadji P, Subar M, Abreu P, Thomsen T, Banke-Bochita J. Effects of steroidal and nonsteroidal aromatase inhibitors on markers of bone turnover in healthy postmenopausal women. *Breast Cancer Res.* 2007; 9(4):R52. [PubMed: 17692126]
 137. Jung K, Lein M, Stephan C, Von Hosslin K, Semjonow A, Sinha P, Loening SA, Schnorr D. Comparison of 10 serum bone turnover markers in prostate carcinoma patients with bone metastatic spread: diagnostic and prognostic implications. *Int J Cancer.* 2004; 111(5):783–791. [PubMed: 15252851]
 138. Oremek G, Sauer-Eppel H, Klepzig M. Total procollagen type 1 amino-terminal propeptide (total PINP) as a bone metastasis marker in gynecological carcinomas. *Anticancer Res.* 2007; 27(4A):1961–1962. [PubMed: 17649805]
 139. Joerring S, Jensen LT. Changes in collagen metabolites in serum after cemented hip and knee arthroplasty. *Arch Orthop Trauma Surg.* 1993; 112(3):139–141. [PubMed: 8323844]
 140. Eriksen EF, Charles P, Melsen F, Mosekilde L, Risteli L, Risteli J. Serum markers of type I collagen formation and degradation in metabolic bone disease: correlation with bone histomorphometry. *J Bone Miner Res.* 1993; 8(2):127–132. [PubMed: 8442431]
 141. Celeste AJ, Rosen V, Buecker JL, Kriz R, Wang EA, Wozney JM. Isolation of the human gene for bone gla protein utilizing mouse and rat cDNA clones. *EMBO J.* 1986; 5(8):1885–1890. [PubMed: 3019668]
 142. Price PA, Urist MR, Otawara Y. Matrix Gla protein, a new gamma-carboxyglutamic acid-containing protein which is associated with the organic matrix of bone. *Biochem Biophys Res Commun.* 1983; 117(3):765–771. [PubMed: 6607731]
 143. Price PA, Nishimoto SK. Radioimmunoassay for the vitamin K-dependent protein of bone and its discovery in plasma. *Proc Natl Acad Sci U S A.* 1980; 77(4):2234–2238. [PubMed: 6966406]
 144. Ivaska KK, Hentunen TA, Vaaranemi J, Ylipahkala H, Pettersson K, Vaananen HK. Release of intact and fragmented osteocalcin molecules from bone matrix during bone resorption in vitro. *J Biol Chem.* 2004; 279(18):18361–18369. [PubMed: 14970229]
 145. Price PA, Williamson MK, Lothringer JW. Origin of the vitamin K-dependent bone protein found in plasma and its clearance by kidney and bone. *J Biol Chem.* 1981; 256(24):12760–12766. [PubMed: 6975778]
 146. Delmas PD, Wilson DM, Mann KG, Riggs BL. Effect of renal function on plasma levels of bone Gla-protein. *J Clin Endocrinol Metab.* 1983; 57(5):1028–1030. [PubMed: 6604733]
 147. Gundberg CM, Markowitz ME, Mizruchi M, Rosen JF. Osteocalcin in human serum: a circadian rhythm. *J Clin Endocrinol Metab.* 1985; 60(4):736–739. [PubMed: 3871789]

148. Seibel MJ. Biochemical markers of bone turnover: part I: biochemistry and variability. *Clin Biochem Rev.* 2005; 26(4):97–122. [PubMed: 16648882]
149. Pedersen BJ, Schlemmer A, Hassager C, Christiansen C. Changes in the carboxyl-terminal propeptide of type I procollagen and other markers of bone formation upon five days of bed rest. *Bone.* 1995; 17(1):91–95. [PubMed: 7577164]
150. Lian J, Stewart C, Puchacz E, Mackowiak S, Shalhoub V, Collart D, Zambetti G, Stein G. Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression. *Proc Natl Acad Sci U S A.* 1989; 86(4):1143–1147. [PubMed: 2784002]
151. Schwab AM, Granholm S, Persson E, Wilkes B, Lerner UH, Conaway HH. Stimulation of resorption in cultured mouse calvarial bones by thiazolidinediones. *Endocrinology.* 2005; 146(10):4349–4361. [PubMed: 15994344]
152. Fraser JD, Price PA. Induction of matrix Gla protein synthesis during prolonged 1,25-dihydroxyvitamin D3 treatment of osteosarcoma cells. *Calcif Tissue Int.* 1990; 46(4):270–279. [PubMed: 2108798]
153. Herrmann M, Muller M, Scharhag J, Sand-Hill M, Kindermann W, Herrmann W. The effect of endurance exercise-induced lactacidosis on biochemical markers of bone turnover. *Clin Chem Lab Med.* 2007; 45(10):1381–1389. [PubMed: 17727311]
154. Thompson Z, Miclau T, Hu D, Helms JA. A model for intramembranous ossification during fracture healing. *J Orthop Res.* 2002; 20(5):1091–1098. [PubMed: 12382977]
155. Uusitalo H, Rantakokko J, Ahonen M, Jamsa T, Tuukkanen J, KaHari V, Vuorio E, Aro HT. A metaphyseal defect model of the femur for studies of murine bone healing. *Bone.* 2001; 28(4):423–429. [PubMed: 11336924]
156. Price PA, Parthemore JG, Deftos LJ. New biochemical marker for bone metabolism. Measurement by radioimmunoassay of bone GLA protein in the plasma of normal subjects and patients with bone disease. *J Clin Invest.* 1980; 66(5):878–883. [PubMed: 6968755]
157. Dias IR, Viegas CA, de Azevedo JT, Costa EM, Lourenco P, Rodrigues A, Cabrita AS. Assessment of markers of bone formation under controlled environmental factors and their correlation with serum minerals in adult sheep as a model for orthopaedic research. *Lab Anim.* 2008; 42(4):465–472. [PubMed: 18782825]
158. Nyman MT, Paavolainen P, Forsius S, Lamberg-Allardt C. Clinical evaluation of fracture healing by serum osteocalcin and alkaline phosphatase. *Ann Chir Gynaecol.* 1991; 80(3):289–293. [PubMed: 1759799]
159. Kurdy NM. Serology of abnormal fracture healing: the role of PIIINP, PICP, and BsALP. *J Orthop Trauma.* 2000; 14(1):48–53. [PubMed: 10630803]
160. Lotz J, Gaertner T, Hahn M, Prellwitz W. Collagen type I metabolism after bone surgery. *Arch Orthop Trauma Surg.* 1999; 119(3–4):212–216. [PubMed: 10392522]
161. Kurdy NM, Bowles S, Marsh DR, Davies A, France M. Serology of collagen types I and III in normal healing of tibial shaft fractures. *J Orthop Trauma.* 1998; 12(2):122–126. [PubMed: 9503302]
162. Joerring S, Krogsgaard M, Wilbek H, Jensen LT. Collagen turnover after tibial fractures. *Arch Orthop Trauma Surg.* 1994; 113(6):334–336. [PubMed: 7833212]
163. Obrant KJ, Merle B, Bejui J, Delmas PD. Serum bone-gla protein after fracture. *Clin Orthop Relat Res.* 1990; (258):300–303. [PubMed: 2394055]
164. Garnero P, Vergnaud P, Hoyle N. Evaluation of a fully automated serum assay for total N-terminal propeptide of type I collagen in postmenopausal osteoporosis. *Clin Chem.* 2008; 54(1):188–196. [PubMed: 17998267]
165. Ivaska KK, Gerdhem P, Akesson K, Garnero P, Obrant KJ. Effect of fracture on bone turnover markers: a longitudinal study comparing marker levels before and after injury in 113 elderly women. *J Bone Miner Res.* 2007; 22(8):1155–1164. [PubMed: 17488197]
166. Ingle BM, Hay SM, Bottjer HM, Eastell R. Changes in bone mass and bone turnover following ankle fracture. *Osteoporos Int.* 1999; 10(5):408–415. [PubMed: 10591839]
167. Ingle BM, Hay SM, Bottjer HM, Eastell R. Changes in bone mass and bone turnover following distal forearm fracture. *Osteoporos Int.* 1999; 10(5):399–407. [PubMed: 10591838]

168. Christensen B, Dyrberg E, Aagaard P, Enejhjem S, Krogsgaard M, Kjaer M, Langberg H. Effects of long-term immobilization and recovery on human triceps surae and collagen turnover in the Achilles tendon in patients with healing ankle fracture. *J Appl Physiol*. 2008; 105(2):420–426. [PubMed: 18403455]
169. Komatsu DE, Brune KA, Liu H, Schmidt AL, Han B, Zeng QQ, Yang X, Nunes JS, Lu Y, Geiser AG, Ma YL, Wolos JA, Westmore MS, Sato M. Longitudinal in vivo analysis of the region-specific efficacy of parathyroid hormone in a rat cortical defect model. *Endocrinology*. 2009; 150(4):1570–1579. [PubMed: 19022894]
170. Veitch SW, Findlay SC, Hamer AJ, Blumsohn A, Eastell R, Ingle BM. Changes in bone mass and bone turnover following tibial shaft fracture. *Osteoporos Int*. 2006; 17(3):364–372. [PubMed: 16362144]
171. Chevrel G, Limouzin A, Garnerio P, de Montalivet C, Loubier D. Fractures and handicap in an adult population: A clinical study. *Joint Bone Spine*. 2007; 74(6):584–589. [PubMed: 17920322]
172. Zanker CL, Swaine IL. Responses of bone turnover markers to repeated endurance running in humans under conditions of energy balance or energy restriction. *Eur J Appl Physiol*. 2000; 83(4–5):434–440. [PubMed: 11138586]
173. Cramer RM, Langberg H, Teisner B, Magnusson P, Schroder HD, Olesen JL, Jensen CH, Koskinen S, Suetta C, Kjaer M. Enhanced procollagen processing in skeletal muscle after a single bout of eccentric loading in humans. *Matrix Biol*. 2004; 23(4):259–264. [PubMed: 15296940]
174. Haining SA, Atkins RM, Guillard-Cumming DF, Sharrard WJ, Russell RG, Kanis JA. Vitamin D metabolites in patients with established non-union of fracture. *Bone Miner*. 1986; 1(3):205–209. [PubMed: 3509893]
175. Southwood LL, Frisbie DD, Kawcak CE, McIlwraith CW. Evaluation of serum biochemical markers of bone metabolism for early diagnosis of nonunion and infected nonunion fractures in rabbits. *Am J Vet Res*. 2003; 64(6):727–735. [PubMed: 12828259]
176. Gineyts E, Cloos PA, Borel O, Grimaud L, Delmas PD, Garnerio P. Racemization and isomerization of type I collagen C-telopeptides in human bone and soft tissues: assessment of tissue turnover. *Biochem J*. 2000; 345(Pt 3):481–485. [PubMed: 10642505]

FIGURE 1.

Type I collagen synthesis. Illustration of the posttranscriptional and posttranslational events during collagen type I fibrillogenesis. After the induction of collagen type I synthesis, hydroxylation and glycosylation characterize the early phase of procollagen type I synthesis within the endoplasmic reticulum (1). Two $\alpha 1$ -polypeptide chains and one $\alpha 2$ -chain are assembled and the triple-helical procollagen type I is formed (2). Following exocytosis (3), the amino-terminal and carboxy-terminal propeptide extensions are extracellularly cleaved by specific zinc-dependent proteinases (4). During collagen maturation (5), pyridinium cross-links are formed and enable staggering of the triple-helical molecules following a hexagonal structural and amino-to-carboxy-terminal orientation pattern. The cleaved components (PINP and partially PICP) are released into the extracellular matrix and interstitial space (6). Col-I-Fib, collagen type I fibril; CP, C-proteinase; ER, endoplasmic reticulum; NC, nucleus; NP, N-proteinase; PC-I: pro-collagen type I; PICP, procollagen I C-terminal propeptide; PINP, procollagen I N-terminal propeptide.

FIGURE 2.

Stages of endochondral bone healing. (1) Radiographic image of an acute fracture of the femur shaft with intramedullary fixation in a mouse (left). The square (black) indicates the representative area of the bone healing stage within the specimen (inflammation phase). The illustration (right) delineates the inflammatory phase of fracture healing. Cortical and spongy bone are interrupted. The periost is disrupted, but can also be intact. Mesenchymal stem cells invade the injury zone, which is surrounded by hematoma. (2) Radiographic image at 1 week after fracture (left). Minimal callus formation is visible. The square (black) indicates the zone of healing, which is illustrated on the right (reparation phase). The hematoma has been resolved and a granulation tissue has developed including fibroblasts. Mesenchymal stem cells further differentiate into osteocytes and chondrocytes. Newly formed cartilage consists of collagen type III (especially in the early phase of reparation) and collagen type I. The cartilage matrix is transformed into woven bone. (3) Radiographic image at 2 weeks after fracture (left). Significant amounts of callus are visible, but the fracture is not fully bridged and thus remains unstable. The square (black) indicates the illustrated zone of healing (right, early remodeling phase). Vascular invasion and callus growth characterize this stage. (4) Radiographic image at 23 days after fracture (left). Bridging callus indicates a healed fracture. The square (black) indicates the illustrated zone of healing (right, late remodeling phase). The late stage of endochondral trabecular bone formation is associated with osteoblast and TRAP-positive cell settlement in the marrow cavity, unity of fracture ends, and regeneration of bone marrow space. Ca, Cartilage; CB, cortical bone; CC, chondrocyte; FB, fibroblast; GT, granulation tissue; H, hematoma; MC, medullary cavity; MSC, mesenchymal stem cell; OC, osteocyte; P, periost; WB, woven bone.

FIGURE 3.

Radiographic appearance of fracture healing. (A) Hypertrophic nonunion: anterior-posterior view of a distal diaphyseal fracture of the tibia 2 weeks after a low energy fall in a 55-year-old, brittle, insulin-dependent diabetic female treated with prolonged cast immobilization (A1). The lateral view reveals a hypertrophic nonunion with persistent lucency at the fracture site (arrow) (A2). Radiographic follow-up at 6 months demonstrates worsening varus deformity and excessive callus formation (dotted arrows) in the anterior-posterior view (A3). At 1 year, the radiograph demonstrates healed hypertrophic nonunion after intervention with an intramedullary nail, creating a stable environment to heal the fracture without disruption of the blood supply (A4). (B) Normal healing with nail stabilization: anterior-posterior view of a severe open multi-fragmented segmental diaphyseal fracture of the tibia and fibula after a motorcycle accident injury in a 45-year-old male nonsmoker (B1). Healed tibial fracture in the asymptomatic patient (arrows: bridging bone) at 2-year follow-up (B2). (C) In comparison with panel B, radiographic follow-up (anterior-posterior view) at 12 months after operative fixation with an intramedullary nail in a 45-year-old male smoker after a motorcycle crash, demonstrating absent callus formation or remodeling (straight arrows). Secondary to instability and avascularity, haloing at the proximal fragment in close proximity to the implant is noted (dotted arrows).

FIGURE 4. PINP and PICP formation. Both the N-propeptide and the C-propeptide are extracellularly cleaved from the fibril by specific zinc-dependent proteinases. PICP, procollagen I C-terminal propeptide; PINP, procollagen I N-terminal propeptide.