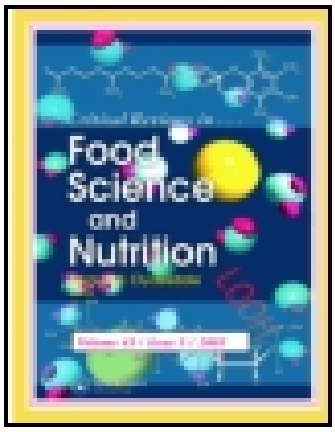

Critical Reviews in Food Science and Nutrition
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/bfsn20

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Audrey Daneault[a], Véronique Coxam[a] & Yohann Wittrant[a]
[a] INRA, St Genes Champanelle, France, Email: ,
Accepted author version posted online: 15 May 2015.

To cite this article: Audrey Daneault, Véronique Coxam & Yohann Wittrant (2015): Biological Effect of Hydrolyzed Collagen on Bone Metabolism, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2015.1038377

To link to this article: http://dx.doi.org/10.1080/10408398.2015.1038377

BIOLOGICAL EFFECT OF HYDROLYZED COLLAGEN ON BONE METABOLISM

Mrs Audrey Daneault[1], Véronique Coxam[1], Dr Yohann Wittrant[*]

[1]INRA, St Genes Champanelle, France, Email: audrey.daneault@clermont.inra.fr, veronique.coxam@clermont.inra.fr

[*]Dr Yohann Wittrant (Corresponding Author) INRA, Centre de Recherche Clermont-Ferrand/Theix, St-Genes Champanelle, 63122 France, Email: yohann.wittrant@clermont.inra.fr

Abstract

Osteoporosis is a chronic and asymptomatic disease characterized by low bone mass and skeletal microarchitectural deterioration, increased risk of fracture and associated co-morbidities most prevalent in the elderly. Due to an increasingly aging population, osteoporosis has become a major health issue requiring innovative disease management. Proteins are important for bone by providing building blocks and by exerting specific regulatory function. This is why adequate protein intake plays a considerable role in both bone development and bone maintenance. More specifically, since an increase in the overall metabolism of collagen can lead to severe dysfunctions and a more fragile bone matrix and because orally administered collagen can be digested in the gut, cross the intestinal barrier, enter the circulation and become available for metabolic processes in the target tissues, one may speculate that a collagen-enriched diet provides benefits for the skeleton. Collagen-derived products such as gelatin or hydrolyzed collagen (HC) are well acknowledged for their safety from a nutritional point of view, however what is their impact on bone biology? In this manuscript, we critically review the evidence from literature for an effect of HC on bone tissues in order to determine whether HC may represent a

relevant alternative in the design of future nutritional approaches to manage osteoporosis prevention.

Abbreviation: Hydrolyzed Collagen (HC)

Keywords: Collagen · Hydrolyzed collagen · Bone · Osteoporosis

1. A need for alternative osteoporosis treatments

Osteoporosis is considered as a major cause of morbidity, disability and as an important contributor to medical care costs in many regions of the world. Its prevalence increases with age and the disease is twice more common in women than in men because of the hormonal changes that occur during menopause (Kanis, 1994). It has been defined as a skeletal disorder characterized by a low bone mineral density and micro-architectural alterations of bone tissue predisposing to an increased risk of fracture (1993; NIH, 2000). Several drugs are available for the treatment of osteoporosis such as bisphosphonates or parathyroid hormone derivates. However, it has been highlighted that compliance to such therapy is usually poor and that the benefit does not continue after the end of treatment. This is why there is an increasing rationale to focus on early prevention to avoid or delay limitations of skeletal functions, rather than to curative strategies. However, classical prophylaxis with hormone replacement therapy is restricted due to concerns about an increased risk for cancer and cardiovascular disease. This is why health professionals strongly advocate the implementation of new strategies with proven scientific and clinical value for the prevention of osteoporosis (Coxam, Davicco, & Demigne, 2008). In this light, food has multiple assets for good compliance. Over the past 30 years research in nutrition has led to an exciting progress supporting the hypothesis that dietary intervention, including dietary supplements, can modulate specific target functions in the body and thus reduce the risk of disease. In this line dietary intervention may offer an effective means to deal with the problem of osteoporosis and its consequential health costs. A nutritional approach has been shown to be a cost-effective way of reducing calcium and vitamin D insufficiency, and thereby improving bone health and reducing fracture risk (Lotters et al., 2013).

The primary goal of a nutritional strategy for the prevention of bone loss is to provide a sufficiently bioavailable amount of constitutive elements such as calcium, proteins as well as nutrients endowed with specific bone sparing properties (proteins, some fatty acids, micronutrients...) (Coxam et al., 2008; Nieves, 2013). Based on this concept and because proteins play a major role in bone by providing building blocks and by exerting specific regulatory functions collagen may provide a new option for aging consumers to maintain good health. Nevertheless, the scientists need to provide a high level of proof based on clinical trials, preclinical investigations and mechanistic studies to establish a health claim.

2. Collagen and bone biology

2.1. Collagen structure is associated with bone mechanical properties

Collagen comprises of three polypeptide strands (alpha-chains) which form a unique triple-helical structure (Fig. 1). To wind into a triple helix, the chains must contain glycine as every third residue thus presenting the repeating structure Gly-X-Y (Exposito, Valcourt, Cluzel, & Lethias, 2010), in which X and Y are mainly proline (Pro) and hydroxyproline (Hyp) (Gelse, 2003). The resulting Gly-Pro-Hyp triplet is the most frequent (10.5%) (Ramshaw, Shah, & Brodsky, 1998). In addition, the amino-acids Lys, Gln and Arg show a periodic distribution of 18 residues (Ottani, Martini, Franchi, Ruggeri, & Raspanti, 2002; Ramshaw et al., 1998). Collagens represent 30% of the total protein mass in the body (Ricard-Blum, 2011) and are therefore the most abundant proteins in mammals. They are the major structural element in the extracellular matrix of all connective tissues, including bone where they represent about 80% of the total protein (Tzaphlidou, 2005). While the mineral content mainly determines bone stiffness and rigidity, collagens provide skeletal toughness. Basically, they form the scaffold for the

attachment of cells and the anchorage of macromolecules, defining the shape of the tissue. Collagen fibers in bone are organized in concentric layers providing maximal resistance against torsional and compressive stress (Bailey, 2001). Within the fibers, the collagen molecules are precisely aligned in a quarter-staggered end-overlap manner. This arrangement provides holes within the fiber for the nucleation of calcium apatite crystals.

As a matter of fact, the term "collagen" comprises a large and still growing family of proteins. They all share the same feature: a right-handed triple helix composed of alpha-chains assembled into a rope-like figure bordered by the C- and N-propeptides (Shoulders & Raines, 2009) (Fig. 1). However, if the average collagen molecule measures 300 nm in length (corresponding to about 1000 amino acids) and 1.5 nm in diameter, the length of the triple helical part varies considerably between the different collagen types (Exposito et al., 2010; Ottani et al., 2002). Collagen types, their distribution and composition are listed in Table 1S (supplemental data). In bone approximately 95% is type I collagen (a heterotrimer formed by two identical α1(I)- chains and one α2(I)-chain) providing viscoelastic strength, torsional stiffness, and load bearing capacity while also presenting nucleation sites for crystalline deposition. Type II collagen is also involved in bone formation, even though it is mainly found in cartilage (Álvarez et al., 2000; Aszódi, Chan, Hunziker, Bateman, & Fässler, 1998). Types III, V (Kahai, Vary, Gao, & Seth, 2004), VI (Keene, Sakai, & Burgeson, 1991), and X (Rosati et al., 1994) are present in bone at a very low level. Unlike type I, collagen type III fibrils are less ordered, thinner, and always combined with other collagen types. Association of types III and VI are characteristic for some regions of mature bone (for example rat proximal femur) (Luther, Saino, Carter, & Aaron, 2003). The function of type V collagen is not well defined (Niyibizi & Eyre,

1994). Type VI is a microfibrillar collagen which seems to line the matrix surrounding the osteocytes and their canaliculi (Keene et al., 1991). Finally, according to Hjorten *et al.*, type XXVII collagen is found during cartilage calcification and the transition of cartilage into bone during osteogenesis as well as in cartilage modeling during endochondral bone formation (Hjorten et al., 2007).

2.2. Collagen network alterations lead to bone fragility

In bone, collagen plays an important role in the force transmission and tissue structure maintenance. Importantly, it determines the amount of mineral deposition. Thus the capacity of bone to resist mechanical forces and fractures depends not only on the quantity of bone tissue (mineralization) but also on its quality (organization of the collagen framework) (Currey, 2003; Viguet-Carrin, Garnero, & Delmas, 2006).

During aging, changes in the collagen network reduce bone mechanical strength and elasticity, which contributes to the occurrence of osteoporotic fractures (Wang, Shen, Li, & Agrawal, 2002). in postmenopausal osteoporosis there is growing evidence that at the material level, the volume fraction of mineral and the relative amounts of mature and immature collagen crosslinks are affected by the tissue turnover rate, thus contributing to bone fragility (Viguet-Carrin et al., 2006). Indeed, estrogen deficiency has been shown to affect collagen stability by decreasing its maturation rate (Sanada et al., 1978). Luther *et al.* observed a disconnection of the collagen fibers after ovariectomy (Luther et al., 2003). In the same line, Kafantari's group reported structural changes in fibril architecture as well as diameter due to altered crosslinks and hydroxylation in the ovariectomized rat (Kafantari, Kounadi, Fatouros, Milonakis, & Tzaphlidou, 2000). Moreover, in inflammation-mediated osteoporosis, severe alterations were

detected at the ultrastructural level in bone and skin collagen fibrils in rabbits (Fountos, Kounadi, Tzaphlidou, Yasumura, & Glaros, 1998).

Regarding the mechanisms involved in ageing, Knott *et al.* highlighted an increase in the overall metabolism of collagen which may account for impaired post-translational modifications, leading to severe dysfunctions in the collagen network and a more fragile bone matrix (Knott & Bailey, 1998). Altered post-translational modifications hamper the formation of cross-links between collagen molecules based on aldehyde formation from specific telopeptide hydroxylysine or lysine residues (Knott & Bailey, 1998) and include an abnormal increase in lysyl hydroxylation or glycosylation, which are key to sustain the structural and mechanical integrity of the collagen network (M. Saito & Marumo, 2010; Yeowell & Pinnell, 1993). These alterations lead to thinner fibrils and higher bone fragility. Another age-related non-enzymatic modification of collagen is the formation of advanced glycation end products (AGE) via the so-called Maillard reaction, due to the accumulation of reducible sugars in bone tissue (Viguet-Carrin et al., 2006). In addition, racemization (spontaneous conversion of the L-enantiomeric form to the biologically rare D-form) and isomerization (transfer of the peptide backbone from the aspartyl residue alpha-carboxyl group to the side chain beta- or gamma- carboxyl group) occur during aging, resulting in structurally altered forms of the collagen molecule with disrupted function (Viguet-Carrin et al., 2006).

The knowledge of certain genetic diseases further emphasizes the importance of correctly formed collagen. The replacement of just one glycine residue by another amino acid can lead to pathologies such as osteogenesis imperfecta and the Ehlers-Danlos Syndrome which are characterized by bone fragility, weak tendons and thin skin (Gautieri, Buehler, & Redaelli,

2009). Subtypes of the Ehlers-Danlos Syndrome are linked to mutations in type I or type III collagens, lysyl hydroxylase, or procollagen N-proteinase (Yeowell & Pinnell, 1993). Type VI collagen deficiency results in a disorganized collagen arrangement suggesting that collagen type VI contributes to maintain bone mass (Izu et al., 2012). Mutations in COL1A1 coding for the α1(I)-chain) and COL1A2 (coding for the α2(I)-chain) are linked to osteogenesis imperfecta, a group of brittle bone diseases. Further, a polymorphism in the Sp1 binding site of the COL1A1 gene results in the synthesis of altered collagen with a possible association to both decreased bone strength and bone mineral density and has thus been postulated to play a role in osteoporosis (Mann et al., 2001). In summary, mutations in genes that encode individual chains of triple-helical bone collagens as well as in genes encoding proteins involved in the intracellular and extracellular modifications of the molecule are associated with heritable diseases of the skeletal tissues and the development of skeletal abnormalities (Arnold & Fertala, 2013). These data emphasize the major role of collagen quantity and quality in bone remodeling.

3. Collagen in nutrition and food supplements

Collagen-derived ingredients (gelatin and HC) are widely used in food, cosmetic and pharmaceutical industries or tissue engineering thanks to their gelling capacity (gel formation, texturizing, thickening and water binding capacities) as well as their surface (emulsion, foam formation and stabilization, adhesion and cohesion, protective colloid function and film forming capacity) and hydration properties (swelling and solubility). The terms “hydrolyzed gelatin”, “collagen hydrolysate”, “hydrolyzed collagen” or sometimes “collagen peptides” used in publications designate the same product. Gelatin is obtained by a partial thermal hydrolysis of collagen which (partially) separates the chains by destroying the crosslinks (Fig. 2).

Subsequently, gelatin is extracted, purified and dried (Karim & Bhat, 2009). Two types of gelatin with different characteristics can be manufactured. Type A gelatin is produced from acid-treated collagen, while alkali-treatment forms type B gelatin. The extraction process (temperature, time, and pH) can influence the length of the polypeptide chains and the functional properties of the gelatin. This is why the quality of a gelatin preparation depends on the manufacturing method, but also from which species or tissue it is extracted (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). For instance, shark gelatin has different characteristics than pig gelatin (Yoshimura et al., 2000).

To form HC, gelatin is submitted to an enzymatic hydrolysis, the most commonly used proteases being trypsin, chymotrypsin, pepsin, alcalase, properase E, pronase, collagenase, bromelain and papain (Gómez-Guillén et al., 2011). HC is usually presented as a white powder with good solubilisation properties, commonly used as a dietary supplement or included in various foodstuffs. Like for gelatin, HC molecular weight distribution, structure and composition, and subsequently functional properties, depend on the processing conditions as well as the raw material and the specificity of the enzyme used to hydrolyze the gelatin (Denis et al., 2008). The average molecular weight of HC ranges between 2,000 and 6,000 Daltons (Moskowitz, 2000). The most abundant sources of gelatin or HC are derived from mammals such as pig skin (46%), bovine hide (29.4%) and pork and cattle bones (23.1%) (Gómez-Guillén et al., 2011). However, the demand for alternative sources has increased after the bovine spongiform encephalopathy (BSE) crisis and for religious and cultural reasons (Karim & Bhat, 2008; Mhd Sarbon, Badii, & Howell, 2013). Production from non-mammalian species, for instance from

fish, is thus of growing importance (Mhd Sarbon et al., 2013; Nagai & Suzuki, 2000; Singh, Benjakul, Maqsood, & Kishimura, 2011; Zeng et al., 2012).

3.1. Safety

Gelatin, and by extension HC, have been approved as Generally Recognized As Safe (GRAS) by the US Food and Drug Administration (USFDA) Center for Food Safety and Nutrition. Indeed, there is no documented evidence of a deleterious effect from the ingestion of HC other than a rare allergy, sensation of unpleasant taste or feeling of heaviness in the stomach. In a multicenter, randomized, parallel, placebo-controlled clinical trial, of 389 patients who were orally treated with 10g HC or placebo over a period of 6 months, only 12 dropped out due to side effects and among those 9 had received the placebo (Moskowitz, 2000). Comparably, in a multicenter, randomized, parallel, double-blind study carried out on one hundred male and female volunteers aged ≥ 40 years with knee osteoarthritis, HC was well tolerated (Trc & Bohmova, 2011). Recorded adverse events were similar whether the volunteers were given 10g HC daily or glucosamine sulphate for 90 consecutive days (Trc & Bohmova, 2011). HC tolerability has also been assessed in various animal studies. Acute, subacute, mutagenic and teratogenic toxicity analyses have not indicated any health risk. Indeed, Takeda *et al.* studied the acute and subacute toxicity of collagen from bovine derm, showing no marked deleterious effect except for local irritation which was seen only after parenteral administration (U. Takeda et al., 1982). In the same line, Wu *et al.* described the high safety of oral HC administration in a rat model when given 1660 mg/kg body weight per day (corresponding to about 10 times the currently used doses in humans). Notwithstanding, rats could exhibit kidney hypertrophy at a dose of 100 times the recommended daily intake (166 mg/kg body weight per day) (Wu, Fujioka, Sugimoto, Mu, &

Ishimi, 2004). Schauss *et al.* also conducted two acute and subchronic oral toxicity investigations in rats with hydrolyzed chicken sternal cartilage which contains mostly type II collagen (Schauss, Merkel, Glaza, & Sorenson, 2007). With a single dose of 5000 mg/kg, all the animals survived without any major pathological lesions, exhibiting a normal body weight gain throughout the study. Regarding subchronic toxicity, all the animals survived and showed no significant changes in body weight or histopathology, whether they were administered 0, 30, 300 or 1000 mg/day of the test product per kg of body weight for over 90 days. Additionally, the risk for chronic toxic effects was not higher in marine HC-treated rats at concentrations of 2.25%, 4.5%, 9% and 18% (wt/wt) in the diet (equivalent to 1063, 2216, 4609 and 8586 mg/kg·body weight/day for females, and 0907, 1798, 3418, 6658 mg/kg·bw/day for males, respectively), than in those fed the basal rodent diet (Liang et al., 2012). Note however that cardiac arrhythmias have been observed in 3 of 6 subjects receiving 300 kcal/ day as HC (equivalent to 75 g/day, ie all the protein intake in the form of collagen) supplemented with triptophane, calcium, magnesium, phosphorus, potassium, vitamins (Lantigua, Amatruda, Biddle, Forbes, & Lockwood, 1980). Deaths have even been registered in obese adults who were reducing their body weight by means of diets that provided same amounts of collagen or gelatin hydrolysates (300 to 500 kcal/ day) without any supplementation in micronutrients (Van Itallie & Yang, 1984).

3.2. Bioavailability

Orally administered HC are digested in the gut, cross the intestinal barrier, enter the circulation and become available for metabolic processes in the target tissues. Even though HC does not contain all the essential amino acids (tryptophan is not present, and cysteine only in

small amounts), it is often used to supplement other proteins because of its high digestibility, good consumer tolerance and its specific amino-acid content (high Hyp, Pro and Gly) (Iwai et al., 2005; Ohara, Matsumoto, Ito, Iwai, & Sato, 2007). As a matter of fact, ingestion of a protein hydrolysate, as opposed to its intact form, accelerates the digestion and the absorption from the gut, increases postprandial amino acid bioavailability and tends to improve the incorporation rate of dietary amino acids into target tissues (Koopman et al., 2009). This concept was confirmed by Urao *et al.* who found that intestinal permeability followed a different pattern for small molecular weight particles than for large molecules in rabbits, suggesting there may be different mechanisms of intestinal transport for molecules of different size (Urao, Okuyama, Drongowski, Teitelbaum, & Coran, 1997). It has been proposed that HC peptides are only digested to a certain degree within the gastrointestinal tract, with a proportion of intact high-molecular-weight proteins reaching the blood by passing through the enterocyte (transcytosis) at a level of approximately 10% (Moskowitz, 2000) (Fig. 3). Oesser *et al.* demonstrated that 95% of orally applied HC were resorbed within 6 hours from the gastro-intestinal tract of mice (Oesser, Adam, Babel, & Seifert, 1999). Just one hour after oral administration already 47% had been absorbed. Iwai *et al*. provided evidence in humans, that oral ingestion of HC significantly increased the peptide form of Hyp in blood with a maximum level after 1-2 hours and a decrease to half of the maximum level after 4 hours (Iwai et al., 2005).

Subsequent to oral ingestion of HC in rodents and humans, various studies have shown that HC-derived amino acids, as well as di- and tripeptides can be detected in blood as well as in various target tissues such as cartilage (Oesser et al., 1999), skin (Kawaguchi, Nanbu, & Kurokawa, 2012) or kidney (Watanabe-Kamiyama et al., 2010). The major collagen-derived

dipeptide found in plasma is Pro-Hyp (Ichikawa et al., 2010; Iwai et al., 2005; Shigemura et al., 2011). It is highly resistant to hydrolysis and is not digestible by peptidase (Aito-Inoue, Lackeyram, Fan, Sato, & Mine, 2007) like other Hyp-containing peptides (Ohara et al., 2007). In addition, small amounts of other di- and tripeptides such as Ala-Hyp, Ala-Hyp-Gly, Leu-Hyp, Ile-Hyp, Phe-Hyp, Pro-Hyp-Gly, (Iwai et al., 2005) and Gly-Pro-Hyp can be detected (Ichikawa et al., 2010; Watanabe-Kamiyama et al., 2010). Another peptide, Hyp-Gly, was more recently discovered in human plasma upon collagen ingestion (Shigemura et al., 2011; Sugihara, Inoue, Kuwamori, & Taniguchi, 2012). It is noticeable that the average plasma concentration of those peptides is dose-dependent: Hyp-containing peptides reach maximum levels of 6.43, 20.17 and 32.84 nmol/mL following ingestion of 30.8, 153.8 and 384.6 mg doses of HC, respectively (Shigemura et al., 2011; Shigemura, Kubomura, Sato, & Sato, 2014). Moreover, the quantity and structure of such Hyp-containing peptides in human blood after oral administration of HC depends on the source; for example, Ala-Hyp-Gly and Ser-Hyp-Gly were detected only from fish scale gelatin hydrolysate, Ala-Hyp and Pro-Hyp-Gly from fish scale or fish skin gelatin hydrolysates, whereas Leu-Hyp, Ile-Hyp and Phe-Hyp appeared after ingestion of fish and to a lower level porcine skin originated HC (Ohara et al., 2007). Finally, synergistic effects with the food matrix can occur and improve HC absorption, for instance when HC is provided within fermented milk (Walrand, Chiotelli, Noirt, Mwewa, & Lassel, 2008).

3.3. Hydrolyzed collagen reaches the bone tissue

To synthesize a single picogram of collagen type II, more than 1 billion glycine molecules and 620 million proline molecules are required. In the absence of these amino acids, the anabolic phase of collagen metabolism can be impaired (Clark, 2007). Proline and

Hydroxyproline serves to stabilize the collagen triple helix, their structure constrain the rotation of the polypeptide collagen chain and creates and strengthens the helicel characteristics of the molecule. Proline biosynthesis is related to both the citric acid cycle and the urea cycle. In looking at other proline biosynthetic pathways, the arginine / ornithine / glutamic semialdehyde /proline pathway looks the most promising (Barbul, 2008). As a matter of fact, orally consumed HC has not only been shown to be well absorbed in the intestine, but also to accumulate in target tissues. *Kawaguchi et al.* studied the biodistribution of orally administered [^{14}C]Pro-Hyp in rats using autoradiography (Kawaguchi et al., 2012). They observed a wide distribution of radioactivity at 30 min post-dose and a cellular uptake of radioactivity in osteoblasts and osteoclasts as well as in dermal fibroblasts, epidermal cells, synovial cells and chondrocytes after 24 h. In addition, according to Watanabe's group, absorption of low molecular weight HC in the ovariectomized rat was associated with an increased content of the organic substance in bone (Watanabe-Kamiyama et al., 2010). Finally, Barbul has shown that during the early phases of wound healing, wound fluid proline levels are at least 50% higher than plasma levels, suggesting active import of proline into the wound (Barbul, 2008).

4. Hydrolyzed collagen affects bone biology

4.1. Evidence from animal models

Growth models: young growing rats are potential models to study factors that can influence bone mass accrual and thereby affect peak bone mass (table 1). In growing male rats, HC supplementation has been described to promote the development of long bones (Xu, Han, & Li, 2010). The reported increase in size, dry weight, ash weight, bone mineral density and both stiffness and toughness of femurs was likely related to an increased osteoblast activity rather than

a decreased rate of bone resorption, since higher serum osteocalcin and bone-specific alkaline phosphatase (BALP) content was observed with no significant difference in N-terminal telopeptide of type I collagen (NTX). Leem *et al.* confirmed a dose-dependent effect of a selected HC with a molecular weight of <3 kDa on longitudinal bone growth and height of the growth plate in adolescent male rats, whereas gelatin as such failed to produce the same effect (Leem, Lee, Jang, & Kim, 2013). Insulin like growth factor -1 (IGF1) and bone morphogenetic protein-2 were highly expressed in the growth plate in the treated group animals. Accordingly, Takeda *et al.* demonstrated that moderate HC consumption (20% protein in the diet of which 30% was HC) increased bone mass during growth in rats and that running exercise further promoted the effect. No further beneficial effect on bone mass was elicited with a higher HC intake (40% protein in the diet of which 30% was HC) (S. Takeda, Park, Kawashima, Ezawa, & Omi, 2013). Finally, in the work published by Wu et al. (Wu et al., 2004), carried out in growing male rat, collagen peptides given at amounts equal to the currently used doses in humans (166 mg/kg body weight per day) or at a 10 or even a 100 higher dose (1660 or 16660 mg/kg body weight per day), bone mineral density of the femur was significantly higher in the animals given the highest dose.

Bone loss models: Most of the studies set up to study the effect of HC on bone loss have been carried out in young OVX animals (table 1). Although, the only small animal model recommended by the FDA (Thompson, Simmons, Pirie, & Ke, 1995) for preclinical evaluation of postmenopausal bone loss is the aged OVX rat model because in marked contrast to postmenopausal women, growing rodents have very little, if any, bone remodeling (Erben, 1996), young growing rats can provide useful information on the short-term effects of drugs on

bone resorption, calcium kinetics and balance, or calciotropic hormone levels. They can also be used to evaluate the effects of interventions aimed at increasing osteoblastic recruitment and bone formation (Bonjour, Ammann, & Rizzoli, 1999). As a matter of fact, ovariectomized (OVX) rodents are currently used as animal models to study postmenopausal osteoporosis. Estrogen deficiency results in disorganized bone collagen fibrils of smaller diameter in both trabecular and cortical bone (Garcia-Moreno et al., 1995). In inflammation-mediated osteoporosis (similar to senile osteoporosis), severe alterations at the ultrastructural level in bone and skin collagen fibrils were detected in rabbits (Fountos et al., 1998). A growing body of evidence demonstrates the potential of collagen intake to prevent bone loss in models of estrogen deficiency.

Nomura *et al.* demonstrated the efficacy of shark skin gelatin to increase type I collagen and glycosaminoglycan content as well as bone mineral density in the femur of OVX rats to a level comparable in the sham operated group (Nomura, Oohashi, Watanabe, & Kasugai, 2005). In the same line, Han and colleagues tested cod gelatin for 90 days in 3-month old female Sprague-Dawley OVX rats observing a preserved femoral neck bone mineral density and trabecular microarchitectural properties in OVX rats fed a gelatin compared to a control diet (2009). The beneficial effect was partly attributed to a significant reduction of pro-inflammatory cytokines (IL-1beta, IL-6 and TNF-alpha) and a decreased urinary excretion of resorption markers (NTX, C telopeptides of type I collagen (CTX) and deoxypyridinoline). As mentioned above, HC ingestion can increase the content of organic substance in bone (Watanabe-Kamiyama et al., 2010). In OVX rats, HC supplementation at a level 10 times higher than the human recommendations (i.e. 10g/day) unequivocally contributed to the conservation of vertebral mass,

protein content (including osteocalcin) and mechanical strength, not seen when gelatin was used as a supplement (De Almeida Jackix, Cuneo, Amaya-Farfan, de Assuncao, & Quintaes, 2010). In the same experimental model, Kim *et al.* observed a prevention of trabecular bone loss and improved microarchitecture of the lumbar vertebrae (H. K. Kim, Kim, & Leem, 2013). Finally, Guillerminet *et al.* demonstrated that HC administration to 3- month old OVX C3H/HeN mice increased bone mineral density and bone strength (Guillerminet et al., 2010). The fact that plasma concentrations of CTX were lower while BALP levels were higher under HC treatment suggested that collagen can improve bone remodeling. These data allow to test for evidence of heterogeneity of bone turnover in such a condition of bone loss, and to attempt to devise an 'uncoupling index' by using the relationship between bone-specific biochemical markers of bone formation and bone resorption. Indeed, where turnover markers are reported, bone formation and degradation markers should always be reported in tandem (Eastell et al., 1993). In the present case, increased bALP levels, while CTX decreased may indicate a net benefit to bone.

That is, it cannot be determined whether bone formation increased to a greater degree than resorption, suggesting a net benefit to bone, or to a lesser degree, suggesting net harm to bone, or to a similar degree, suggesting bone turnover remains tightly coupled.

A second study by the same group showed that the HC administration for 3 or 6 months significantly prevented bone loss in OVX mice (Guillerminet et al., 2012). The authors further demonstrated that HC ingestion for 3 months is as efficient as raloxifene to protect 3-month-old OVX mice from bone loss. Such a bone sparing effect was also seen as soon as 1 month post-surgery in a follow-up study (Daneault, Coxam, Fabien Soulé, & Wittrant, 2014). Finally, in a mice model of protein undernutrition, have shown that gelatin has differential effects on bone

mineral density compared to casein (6% casein + 4% gelatin having a greater effect than a 10% casein diet) (Koyama et al., 2001).

Bone healing models: Tsuruoka *et al.* have shown that oral administration of HC to rats with femur damage accelerated the fracture healing (Tsuruoka, Yamato, Sakai, Yoshitake, & Yonekura, 2007) (table 1). Accordingly, a 3-week oral supplementation with High Advanced-Collagen Tripeptide (HACP), a soluble powder containing about 20% of Gly-X-Y was beneficial for the bone healing process after a cortical bone defect in rats (Hata et al., 2008).

Altogether these results from preclinical models provide a solid body of evidence that HC has a promising potential to maintain a balanced bone turnover in different physiological settings (growth, bone loss, healing) by promoting bone formation (the ratio of bone formation to resorption biomarkers being been used to represent the state of bone turnover). In those studies, the significant difference in such a ratio denotes an improvement in bone turnover in favor of bone formation resulting from HC supplementation. Consequently, like postulated by Elam et al. (Elam et al., 2014), HC may serve as an effective supplement for preventing bone loss by significantly enhancing the organic substance content of bone. This could be explained by a downregulation of the production of pro-inflammatory molecules such as interleukins- 1b and -6, and tumor necrosis factor-α. Because these cytokines in particular are responsible for upregulation of receptor activator for nuclear factor kappa-B ligand (RANKL) for osteoclast recruitment, this may explain the noteworthy impairment of bone loss

The key emerging question is whether these results can be extrapolated to the human situation.

4.2. Evidence from clinical trials

If HC has already been used as a food supplement to sooth pain in patients suffering from osteoarthritis, to date very few clinical studies have evaluated its effects on bone metabolism (Bagchi et al., 2002; Bruyere, Zegels, et al., 2012; Fujita, Ohue, Fujii, Miyauchi, & Takagi, 2002; Henrotin, Lambert, Couchourel, Ripoll, & Chiotelli, 2011; Moskowitz, 2000; Trc & Bohmova, 2011) (table 2). In most of the studies, HC is applied in association with other compounds like drugs or food supplements (Elam et al., 2014; Hooshmand et al., 2013).

In a first clinical investigation, the effects of calcitonin alone or in combination with a HC-rich diet were studied on bone metabolism in postmenopausal women. The results revealed that a daily ingestion of 10g HC associated with intra-muscular injection of calcitonin (100 UI) twice a week for 24 weeks enhanced and prolonged the effect of the drug as shown by a fall in urinary pyridinoline cross-link levels (Adam, Spacek, Hulejova, Galianova, & Blahos, 1996). Next, Fujita *et al.* evaluated the effect of a daily supplementation with 900 mg absorbable algal calcium, 3.5 g collagen and other matrix components, including glucosamine (Fujita et al., 2002). Urinary excretion of NTX was decreased in the supplemented group. In addition to the calcium-mediated suppression of parathyroid hormone, collagen degradation was reduced by the inhibition of cytokine-induced metalloproteinase release, including collagenase. Consistently, another study reported, that in osteopenic post-menopausal women consumption of 5 g calcium/collagen mix (containing 500 mg of calcium carbonate and 5 µg vitamin D) for 3 months enhanced bone mass by orienting bone turnover towards formation rather than resorption (increased BALP/TRAP5b ratio), compared to control volunteers (given 500 mg of calcium carbonate and 5 µg vitamin D daily) (Hooshmand et al., 2013). However, in another investigation the daily ingestion of only HC (10 g/d) for 24 weeks in osteopenic post-menopausal

women did not produce any significant effect on bone metabolism as assessed by resorption or formation biomarkers such as osteocalcin and BALP (Cuneo, Costa-Paiva, Pinto-Neto, Morais, & Amaya-Farfan, 2010). The authors noticed that the majority of patients exhibited an excess body weight (it is thus possible that they did not receive a sufficient dose) as well as inadequate calcium intake, which could have been limiting for the HC effect. More recently, Elam et al (2014) reported that long-term calcium collagen chelate supplementation together with vitamin D, may provide protection against excessive bone loss and turnover (for which calcium and vitamin D alone could not prevent), in postmenopausal women (Elam et al., 2014).

Finally, since the bone mass at a given age also depends on the peak bone mass acquired during growth, investigating the effect of HC in children is of interest. Martin-Bautista *et al.* demonstrated in a 4-months randomized double-blind study, that a daily intake of HC (with or without calcium) at key stages of growth and development had a beneficial effect on bone remodeling (Martin-Bautista et al., 2011). The bone formation factors Insulin-like growth factor 1 (IGF1) and BALP where higher in the group receiving HC when compared to the placebo group.

Although the existing data on HC effects on bone health in humans is promising, the Group for the Respect of Ethics and Excellence in Science has comprehensively outlined (Bruyere, Rizzoli, et al., 2012), that further, well designed studies are warranted to strengthen the scientific evidence, also with regard to the pathways that mediate HC effects on bone health.

4.3. Mechanisms involved in collagen effects on bone

Changes in bone cell behavior: Studies investigating the *in vitro* effect of hydrolyzed collagen provide interesting data even though we must stay aware of the limitations of such approaches

(table 3). As a matter of fact, in the body, the bone cells are never exposed to collagen as usually used in these studies. Indeed, digestion of dietary collagen in the gastro intestinal tract is followed by first-pass metabolism during absorption, and bioactive molecules (i.e., proteins, peptides...) will appear in the circulation (Fig. 4). Therefore testing the effect of serum from animals fed specific enriched diets on cellular outcomes should provide better information for evaluation of dietary effect on specific organ. It should be noted that only Tsuruoka et al. (Tsuruoka et al., 2007) considered a physiological form (collagen tripeptide).

Most of the studies investigating the effect of HC on bone cell metabolism have focused on bone forming cells (osteoblasts) (Fig. 5). In 1998, Komori *et al.* reported that bone marrow stromal cells differentiate into osteoblasts when cultured with type 1 collagen matrix (Komori & Kishimoto, 1998). Andrianarivo and collaborators demonstrated concurrent biochemical changes in the human cell line MG-63 in response to type I collagen exposure involving increased specific activity of cell-associated alkaline phosphatase and increased secretion of osteonectin (up to 2.5-fold for each protein) (1992). Using osteoblasts derived from rat calvaria and grown on collagen type I films, Lynch *et al.* defined the critical role of type I collagen in mediating the signaling cascade for the expression of a mature osteoblast phenotype and the mineralization of the extracellular matrix in a physiological manner (Lynch, Stein, Stein, & Lian, 1995). They described the temporal expression of genes characterizing distinct periods of growth and differentiation. During the initial proliferation period, expression of fibronectin, beta 1 integrin, and actin was decreased by 50 to 70% in cells grown on collagen. In contrast, alkaline phosphatase enzyme activity was elevated during the proliferation period, while mRNA levels remained low, suggesting a post-transcriptional regulation. In the postproliferative period,

osteonectin, osteocalcin, and osteopontin were up-regulated. These results strongly support that collagen I from bone extracellular matrix may play an important role in osteoblastic differentiation and phenotypic expression.

Regarding HC, Fu *et al.* observed that salmon skin gelatin hydrolysates were capable to induce cell proliferation, accelerated cell cycle progression and to inhibit cell apoptosis in human hFOB1.19 cells, especially when skin HC were hydrolyzed with papain compared to other proteases (Fu & Zhao, 2013). Kim *et al.* confirmed the dose-dependent effect of HC on human osteoblast proliferation (H. K. Kim et al., 2013; H. K. Kim, Kim, & Leem, 2014a) and recent data from our lab have provided evidence of both enhanced osteoblast differentiation and proliferation as well as improved cell survival and viability by bovine HC (Daneault et al., 2014). In parallel, Liu *et al.* demonstrated that bovine HC promotes osteoblast differentiation and mineralized bone matrix formation (Liu et al., 2014). Accordingly, HC dose-dependently stimulates type I collagen mRNA expression and protein production (H. K. Kim et al., 2014a; Tsuruoka et al., 2007; Yamada, Yoshizawa, et al., 2013) as well as alkaline phosphatase activity (Guillerminet et al., 2010; H. K. Kim et al., 2013; Yamada, Nagaoka, et al., 2013). Incubation of human osteoblasts with 0.1% fish HC increased osteocalcin, osteopontin, BMP-2 and integrin β3 mRNA expression and accelerated matrix mineralization as compared to untreated cells (Yamada, Yoshizawa, et al., 2013). Consequently, this translated into increased calcium disposal or mineralization in either human or murine osteoblasts (Liu et al., 2014; Tsuruoka et al., 2007; Yamada, Nagaoka, et al., 2013; Yamada, Yoshizawa, et al., 2013) (Fig. 5). In addition to an effect on osteoblasts, the impact of HC on osteoclast biology was investigated. A significant inhibition of osteoclast formation and activity in cell lines and in primary cultures was observed

when incubated with bovine and porcine HC (Guillerminet et al., 2010) or with shark protein hydrolysates (Uehara, Takahashi, Watanabe, & Nomura, 2014). Consistently, we recently found a higher OPG/RANKL ratio after incubation of MC3T3 cells with bovine HC reflecting an unfavorable metabolic orientation for osteoclast differentiation (Daneault et al., 2014). Similar to HC, in human osteoblastic MG-63 cells, other peptides such as egg yolk-derived peptides have been shown to stimulate early stages of the osteogenic differentiation via the MAPK/ELK1signaling pathway (up-regulation of genes responsible for bone formation such as *ALPL*, *COL1A1*, and *SPP1) and* accelerate mineralization by hastening mineralization initiation, subsequently leading to an increase in the extent of calcium deposition (H. K. Kim, Kim, & Leem, 2014b).

Molecular mechanisms: Interaction of the Asp-Gly-Glu-Ala amino acid domain of type I collagen with the alpha2beta1 integrin receptor on the cell membrane was proven to be an important signal for bone marrow cell differentiation towards an osteoblastic phenotype (Mizuno & Kuboki, 2001). Additionally, HC-induction of the bone-specific transcription factor osterix was associated with the up-reglation of type I collagen expression, thus providing insights into the molecular basis of HC action on osteoblasts (H. K. Kim et al., 2014a; Tsuruoka et al., 2007; Yamada, Yoshizawa, et al., 2013). Bovine HC was shown to stimulate osteoblast differentiation, mineralized bone matrix formation, ALP activity, and osteocalcin production through increased Runx2 expression and activity (Liu et al., 2014). Activation of ERK1/2, JNK1/2, p38, and ELK1 phosphorylation in the human osteoblast cell line MG-63 by HC was correlated with increased COL1A1, alkaline phosphatase, osteocalcin and osteopontin gene expression (H. K. Kim et al., 2014a). Extracellular signal-regulated kinase (ERK) inhibitor abolished the HC-induced

COL1A1 expression, thus supporting the importance of the ERK/MAPK signaling pathway in mediating HC effects on osteoblast cells (H. K. Kim et al., 2013). Furthermore, it cannot be excluded that, due to its richness in aromatic amino-acids (HYP), hydrolyzed collagen can induce IGF1 production which consequently activate a calcium sensing receptor and in turn exert an anabolic effect on bone as previously shown (Conigrave, Brown, & Rizzoli, 2008; Dawson-Hughes, Harris, Rasmussen, & Dallal, 2007). Finally, HC appears to greatly impact osteoblast biology but the mechanisms underlying their action are only partially understood. Besides, the impact of HC on osteoclasts remains to be further investigated.

Other effects of hydrolyzed collagen: In addition to a direct modulation of bone cells, HC has been shown to improve calcium absorption, another very important mechanism for preserving bone capital (G. H. Kim et al., 1998). Indeed, epidemiological, isotopic and meta-analysis studies suggest that dietary protein works synergistically with calcium to improve calcium retention and bone metabolism (Kerstetter, Kenny, & Insogna, 2011). For example, brush border membrane vesicle Ca uptake studies suggest that higher protein intake improves Ca absorption, at least in part, by increasing transcellular Ca uptake (Gaffney-Stomberg et al., 2010). Jung *et al.* isolated fish-bone peptides with a high affinity to calcium and a high content of phosphopeptide (Jung, Lee, & Kim, 2006). Using ovariectomized rats, they observed a higher calcium retention and a preservation of both bone mineral density and bone strength when rodents were supplemented with those peptides. HC from both fish and shrimp were described to contain both, a biologically related calcitonin and/or calcitonin-gene-related peptide (Fouchereau-Peron et al., 1999). Nevertheless this observation requires further investigation with regards to the role of these peptide fragments in bone biology. Besides, HC derived from chicken

bones has been shown to reduce proinflammatory-cytokine production in mice (Zhang et al., 2010) and recent works support natural antioxidative properties of HC peptides (Alemán, Giménez, Pérez-Santin, Gómez-Guillén, & Montero, 2011; Ao & Li, 2012). In addition, as bone tissue function is closely linked to lipid metabolism, it is worth noting that the Pro-Hyp peptide reduces the size of lipid droplets in mouse 3T3-L1 preadipocytes (Minaguchi et al., 2012) and that fish HC was found to affect lipid absorption and metabolism in rats resulting in a lower transient increase of plasma triglycerides and associated inflammation (Masataka Saito, Kiyose, Higuchi, Uchida, & Suzuki, 2009). Finally, it cannot be excluded that immunomodulation can be involved. *In vitro* and *in vivo* studies have shown that certain peptide fractions in fish protein hydrolysates may stimulate the nonspecific immune defense system (Khora, 2013). Indeed, according to Gómez-Guillén et al. collagen and gelatin-derived peptides have numerous bioactivities, among which antimicrobial activity, mineral binding capacity, a lipid-lowering effect, immunomodulatory activity and beneficial effects on skin, bone or joint health (Gómez-Guillén et al., 2011).

5. Conclusion

A growing body of evidence demonstrates that HC owns bioactive properties beneficial for bone tissue, including stimulation of bone forming cells, improvement of calcium absorption, anti-inflammatory and antioxidant capacities. Those properties render HC a new and innovative candidate for putative dietary intervention in the prevention of osteoporosis. Still, many questions remain to be answered: what is the optimal form of HC, what is the optimal dose? To date, we recently started to address these questions and identified that origin and length of hydrolyzed collagen may play an important role in mediating positive action on bone

(unpublished data). In parallel, investigations of the signaling pathways involved in the bone sparing effect are now required to further support these conclusions. Altogether, in the light of the increasing prevalence of osteoporosis related to the worldwide increasing longevity, good candidates for dietary prevention are of particular relevance. As such, HC could offer additional values to calcium and vitamin D, thus responding to the growing demand for primary prevention.

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Table 1 : *In vivo* studies on HC in bone biology

	Model/ species	Number/ groups	Product	Origin	Dose	Duration of treatment	Effect of treatment	Ref
***In vivo* studies**	Growth models	n= 24 male Wistar rats (5-week-old) 4 groups	Collagen peptide MW +/- 5 kDa	Porcine skin	166 mg/kg body weight /day (C1), 1600 mg/kg body weight /day (C10), and 16600 mg/kg body weight /day (C100)	4 weeks	BMD of the femur: C100 group > the other groups Kidneys hypertrophy in the C100 group	Wu et al. 2004
		n=80 Sprague–Dawley (3-week-old) 4 groups	Marine collagen peptides (MCP)	Chum salmon	1125, 2250 or 4500 mg/kg body weight	1 month	Bone size, mineral density,dry weight,ash weight: ↗	Xu et al. 2010
		n= 59 male Wistar rats (5-week-old) 5 groups	HC peptides MW: 1 kDa	Fish	20% casein group vs. 40% casein group vs. 20% HC group vs. 40% HC group	11 weeks	HC intake (20%): ↗ bone mass during growth and exercise period Higher HC intake has no more beneficial effect	Takeda et al. 2013
	Bone loss models	n= 32 SHRSP-Izumo rats (8-week-old) 3 groups: control (SHAM-operated) *vs.* OVX *vs.* OVX with HC	HC MW : 800 Da.	Chicken foot	10% HC in distilled water	20 weeks	↘ bone loss by increasing collagen bone content	Watanabe et al. 2010
		n = 60 femaleWistar rats (1-month-old) 4 groups	Gelatin	Shark skin	10, 20, or 40 mg/100 g of body weight per day	2 weeks	↗ BMD femur epiphysis in treated ovariectomized rats (> SHAM) ↗ contents of type I collagen and glycosaminoglycan	Nomura et al. 2005

						in the epiphysis	
	n= 84 female Wistar rats (3-month-old) 7 groups: control (SHAM) vs OVX vs 5 groups OVX-HC	Gelatin	cod bone	0.375, 0.75, 1.5, 3, 6 mg/kg body weight	90 days	Prevention of BMD loss in proximal tibia and femoral neck at 3 g/kg. ↘ RANKL/OPG mRNA ratio at 3 g/kg and 6 g/kg	Han et al. 2009
	n= 48 female Wistar rats (3-month-old) 6 groups: OVX vs SHAM vs intact +/- gelatin *vs.* Hydrolysed collagen	HC	-	gelatin or HC equivalent from 1000 mg to 100g/day for a 60-kg person	8 weeks	↗ levels of protein and osteocalcin content (HC) preservation of vertebral mass, protein content, and mechanical strength (HC)	De Almeida et al. 2010
	n = 84 female Sprague Dawley rats SHAM vs. OVX: ovariectomized group administrated with 0%−4.0% HC; casein: OVX+casein (1%) group.	HC MW: 3 kDa	Porcine	0%, 0.1%, 0.3%, 1%, 4.0% HC	12 weeks	BMD of whole body and lumbar vertebrae: ↗ (in a dose dependant manner) Femur BMD is not affected by HC treatment.	Kim et al. 2013
	n= 32 female C3H/HeN mice (3-month-old) 3 groups: 8 animals / group control (SHAM-operated) vs. OVX vs. OVX with HC (OVX-CH)	HC MW: 5 kDa	Porcine	Ingestion of 1000 mg HC /kg or and 2500 mg HC/kg body weight .	12 weeks	↗ BMD for OVX (25 g/kg of HC) ↘ CTX level ↗ bone strength (OVX mice 25 g/kg HC)	Guillerminet et al. 2010
	n= 64 female C3H/HeN mice (6-week-old) 2 SHAM groups and 6 OVX groups (3-month-old and 6-month-old)	HC MW: 5 kDa	Porcine	Ingestion of 1000 mg HC /kg or and 2500 mg HC/kg body weight .	26 weeks	↘ bone loss for OVX (25 g/kg of HC) ↗ diameter of the cortical areas of femurs	Guillerminet et al. 2012
	n= 70 female C3H/HeN mice (3-month-old) 2 SHAM groups and 5 OVX groups	HC MW: 5 kDa	Porcine	0 or 2500 mg/kg	12 weeks + 4 additio	HC as efficient as raloxifene	Guillerminet et al. 2012

						nal weeks		
		n= 50 female C3H/HeN mice (9-week-old) 2 groups (SHAM) and 3 groups OVX	HC MW: 2 kDa and 5 kDa	Bovine	2.5% of the diet 15% casein, 17.5% casein or 15% casein plus 2.5% HC	8 weeks	BMD of the femur: ↗ with HC 2kDa	Daneault et al. 2014
	Bone healing models	n= 30 IGS male rats (7-week-old) 3 groups	Collagen tripeptide (Ctp)	Porcine skin	0, 80, or 500 mg/kg/day	12 weeks	Fracture healing acceleration.	Tsuruoka et al. 2007
		n=8 female Wistar/ST rats (11 week-old)	High Advanced-Collagen Tripeptide (HACP)	Hog skin	80mg/2ml/kg body weight	3 weeks	Beneficial effect on bone healing process	Hata et al. 2008

Table 2 : Clinical studies on HC in bone biology

	Model / species	Number/ groups	Product	Origin	Dose	Duration of treatment	Effect of treatment	Ref
Clinical studies	Post meonauposal women	n=94 Calcitonin alone *vs* Calcitonin+HC	HC	-	10g / day	24 weeks	Levels of urinary pyridinoline cross-links: ↗	Adam et al. 1996
		n= 71 Control *vs* HC	HC	-	10g / day	24 weeks	No effect The majority of patients : inadequate calcium intake + excess body weight	Cuneo et al. 2010
		n=29 Control *vs*. CC	Calcium - collagen chelate (CC)	-	5 g of CC + 500 mg calcium carbonate + 5 µg vitamin D (Control: 500 mg calcium carbonate + 5 µg vitamin D)	3 months	bAPL/TRAP5b ratio: ↗ Total BMD: ↗	Hooshmand et al. 2013
		n=39 Control vs. CC	Calcium - collagen chelate (CC)	-	5 g of CC + 500 mg calcium carbonate + 5 µg vitamin D (Control: 500 mg calcium carbonate + 5 µg vitamin D)	12 months	Whole body BMD: □ in CC group ↘ sclérostin ↘ Trap5b ↗ bALP ↗ bALP/Trap5b ratio	Elam et al. 2014

	Pre-pubertal children	n=60 Control (G-I) *vs.* Collagen (G-II) *vs.* Collagen + calcium (G-III)	Partially HC (gelatine)	-	250mL of the product/day Gelatine content is not specified	4 months	serum IGF-1: G-III > G-II or G-I ALP: G-III > G-I ($p < 0.05$)	Martin-Bautista et al. 2011

Table 3 : *In vitro* studies on HC in bone biology

	Model/ species	Number/ groups	Product	Origin	Dose	Duration of treatment	Effect of treatment	Ref
***In vitro* studies**	Osteoblasts	Human osteoblastic MG-63 cells	HC <3 kDa: selected MW: 1.4 kDa	-	0, 10, 25, 50 et 100 µg/mL	-	Cell proliferation: ↗ (dose-dependent) ALP activity: ↗ (dose-dependent) Collagen synthesis and collagen, type1, alpha1 (COL1A1) gene expression: ↗ CH-induced COL1A1 gene expression was completely abolished by extracellular signal-regulated kinase (ERK) inhibitor	Kim et al. 20013
		Primary tissue co-culture	HC MW: 2 and 5 kDa	Bovine, porcine and fish	0.2, 0.5 and 1.0 mg/mL	-	Cell growth : = ALP activity: ↗ (dose-dependent)	Guillermi net et al. 2010
		MC3T3-E1 cells (subclone 4)	HC MW: 2 kDa	Bovine, porcine and fish	0.2, 0.5 and 1.0 mg/mL	-	Absence of cytotoxicity Cell proliferation: ↗ ALP activity : ↗ Ca/P nodule formation: o in MC3T3-E1	Daneault et al. 2014

							cultures	
		hFOB1.19	Collagen tripeptide (Ctp)	Porcine skin	10 µg/ml	-	Calcification: ↗ Type I collagen protein production + its mRNA levels: ↗ Ctp upregulated Osterix.	Tsuruoka et al. 2007
		hFOB1.19	Gelatin hydrolysate degree of hydrolysis of 4,7-13,5%	Chum salmon skin	0, 0.005, 0.01, 0.02, 0.05, 0.1 mg/mL	-	Cell growth proliferation Cell cycle progression acceleration Cell apoptosis inhibition Cell proliferation: ↗ with gelatin prepared with papain protease	Fu et al. 2013
		Human osteoblastic MG-63 cells	HC	-	-	-	Cell proliferation: ↗ ALP activity: ↗ Collagen synthesis: ↗ Calcium deposition: ↗ HC activated ERK1/2, JNK1/2, p38, and ELK1 phosphorylation except cJUN. COL1A1, ALPL, BGLAP, and osteopontin gene expressions : ↗	Kim et al. 2014a

		MC3T3-E1 cells (subclone 4)	Collagen peptide MW: 0.6–2.5 kDa	Bovine	0.1–6 mg/mL Best concentration:3 mg/mL.	-	Cell proliferation: ↗ Percentage of MC3T3-E1 cells in G2/S phase: ↗ Runx2 expression, ALP activity, and OC production:↗ Mineralization: ↗	Liu et al. 2014
		NOS-1 human osteosarcoma	Collagen peptide MW: 3kDa (0.5–10kDa)	Cod skin and bone	ALP: 0.0005, 0.005, 0.05, 0.1 and 0.5% Best concentration:. 0.1%	-	ALP activity : ↗ Cell proliferation: ↗ Osteocalcin, osteopontin, BMP-2 and integrin β3 mRNAs expression: ↗ Osteopontin and integrin β3 expression levels: ↗ Osteocalcin, osteopontin and integrin β3 proteins: ↗ Matrix mineralization: ↗	Yamada, Yoshizawa, et al. 2013
		MC3T3-E1 cells (subclone 4)	Collagen peptide MW: ≈ 2-8 kDa hydroxyproline content:≈5 %	Fish	RT-PCR: 0.05, 0.1, 0.2 and 0.5% (w/v) of FCP Best concentration: 0.2%	-	Lysine hydroxylation,levels of hydroxylysine-aldehyde derived cross-links and cross-link maturation : ↗	Yamada, Nagaoka, et al. 2013

							Collagen synthesis, quality and mineralization: ↗	
	Osteoclasts	Primary tissue co-culture	HC MW: 2 and 5 kDa	Bovine, porcine and fish	0.2, 0.5 and 1.0 mg/mL	-	No effects on cell growth . ALP activity: ↗ (dose-dependent)	Guillermi net et al. 2010

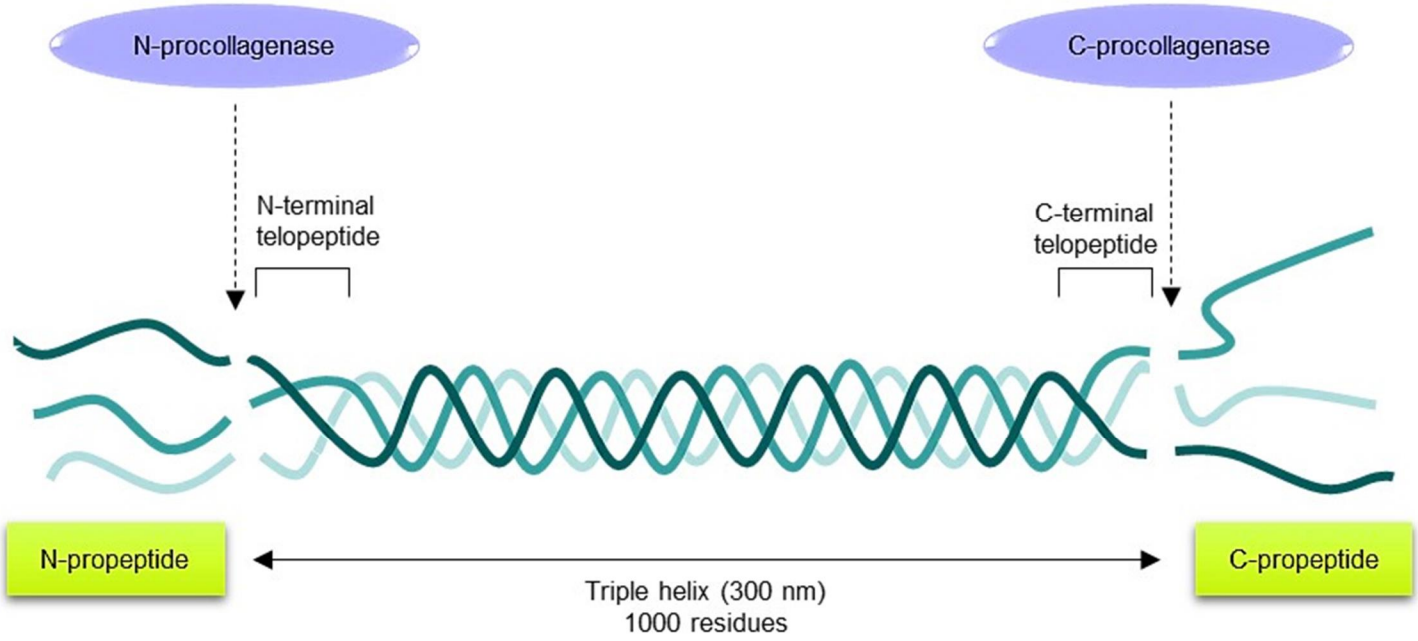

Figure 1: The collagen triple helix

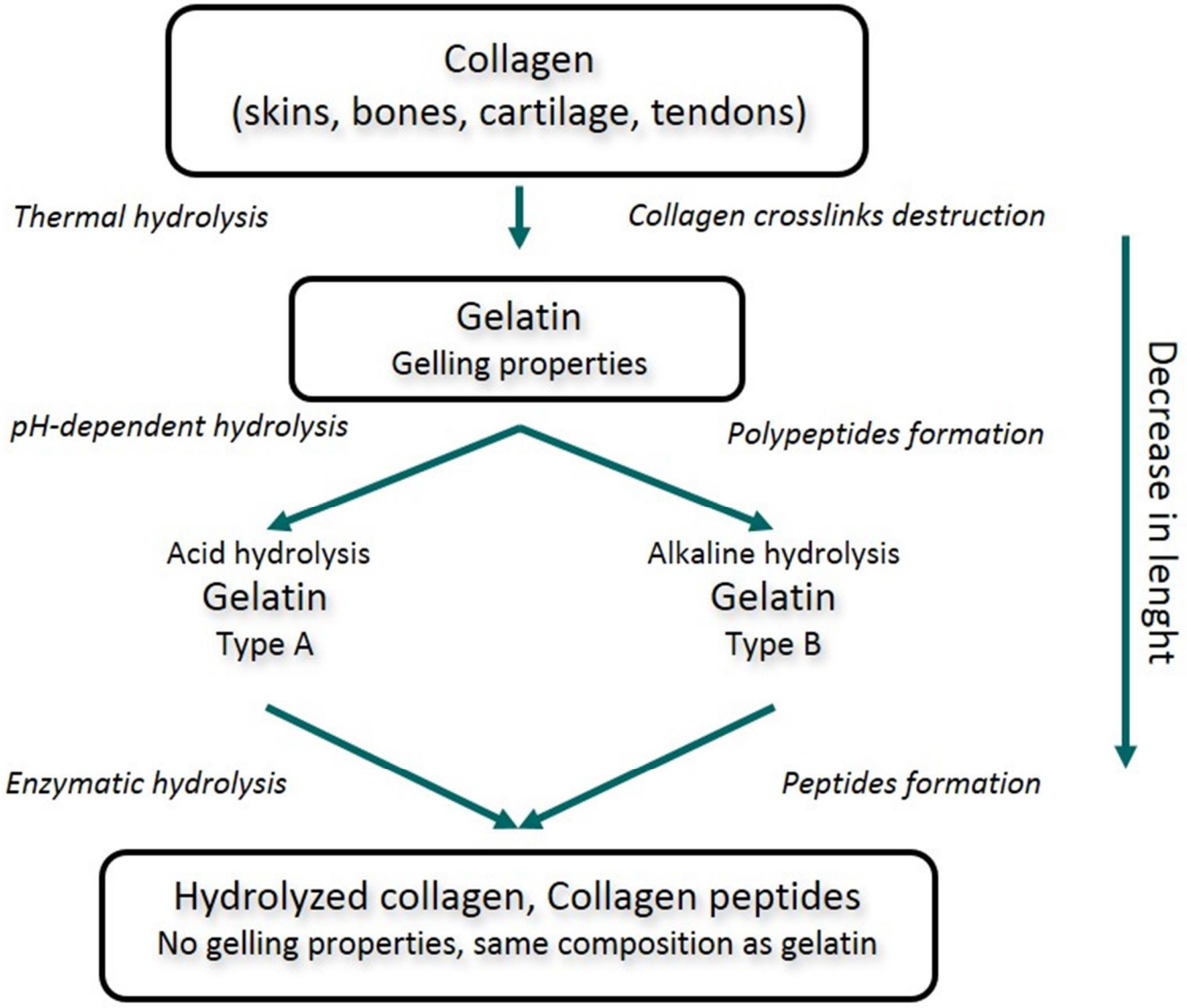

Figure 2: From collagen to gelatin and hydrolyzed collagen

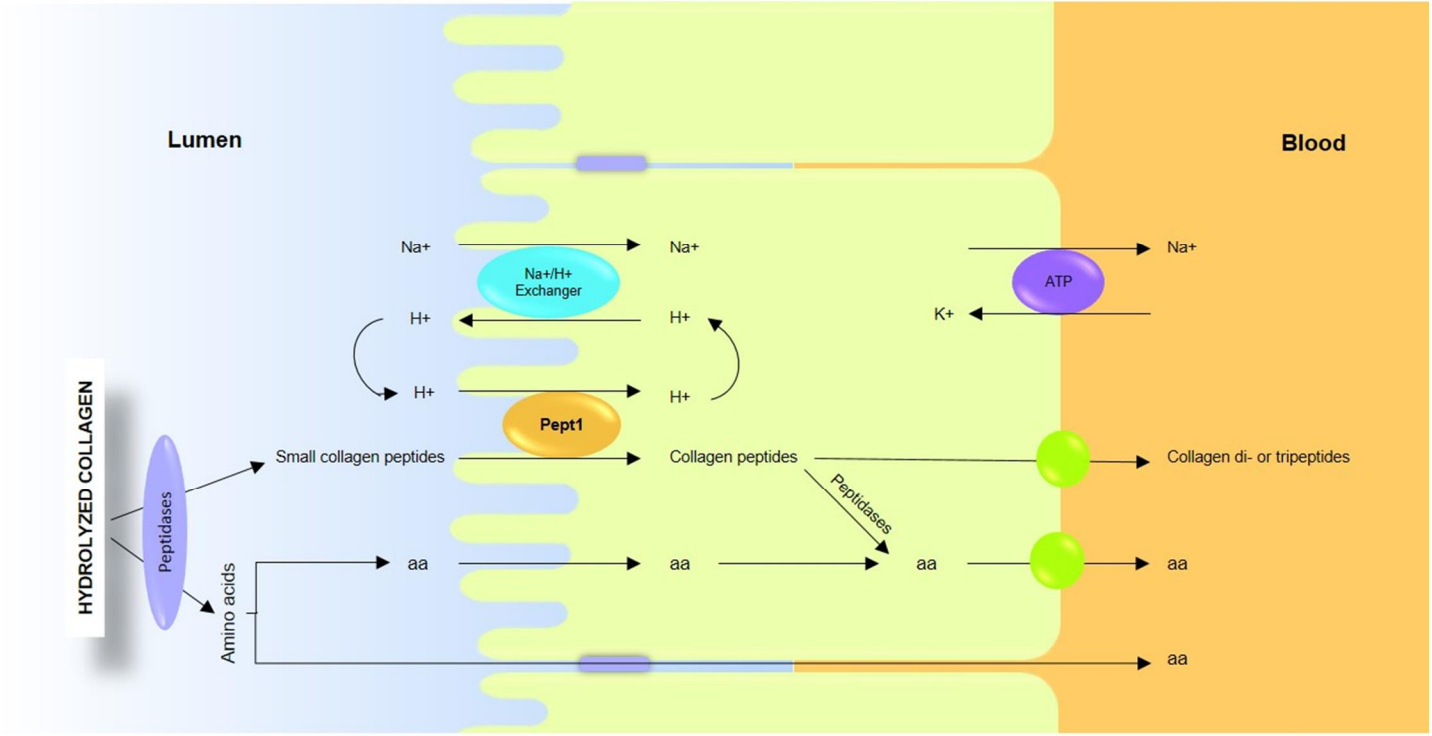

Figure 3: Mechanisms of hydrolyzed collagen intestinal absorption

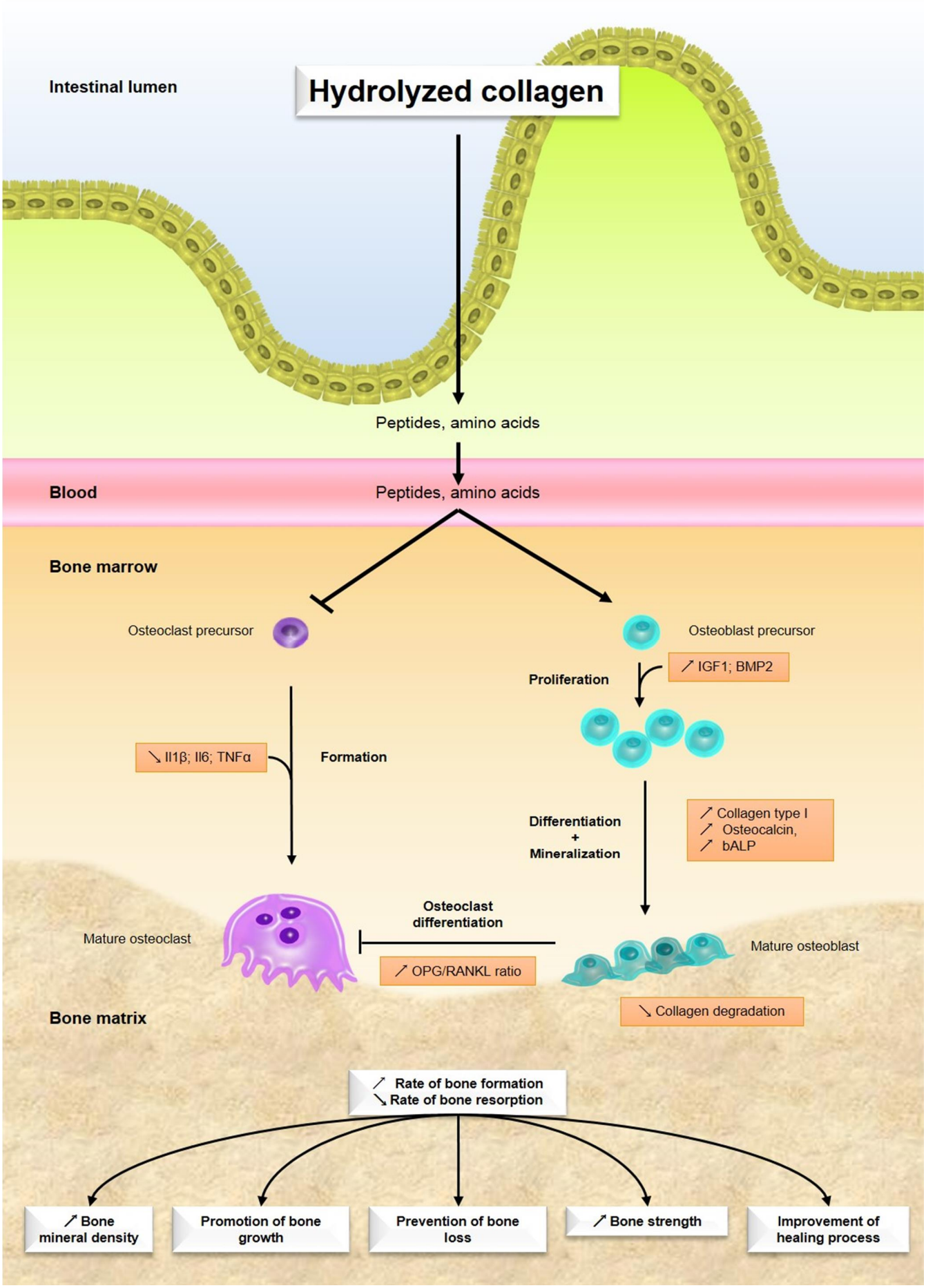

Figure 4: Integrated viewpoint of hydrolyzed collagen effects on bone remodeling process

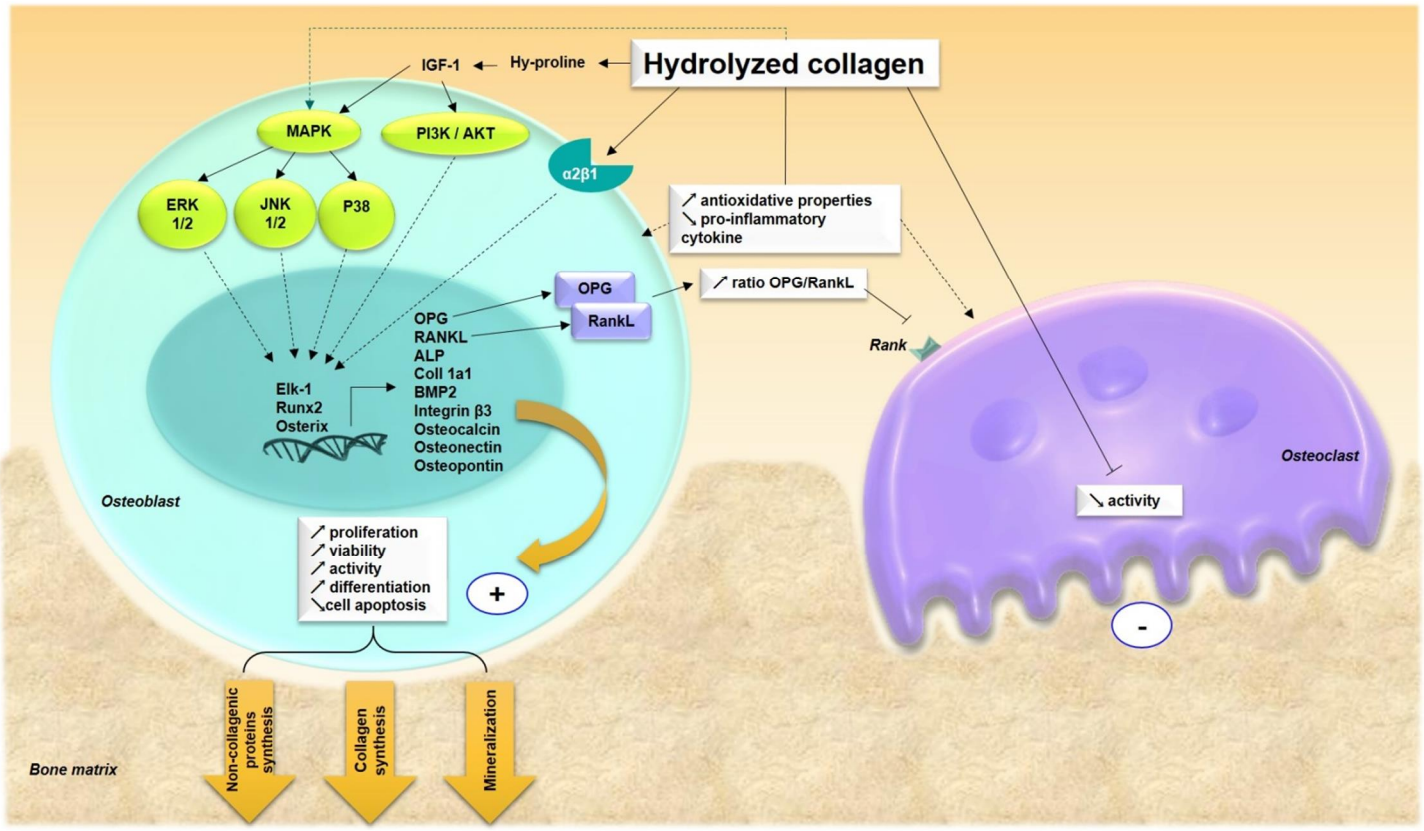

Figure 5: Supposed cellular and molecular mechanisms of hydrolyzed collagen action on bone