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Boning up on DPP4, DPP4 substrates, and DPP4-adipokine interactions : logical reasoning and known facts about bone related effects of DPP4 inhibitors

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Title: Boning up on DPP4, DPP4 substrates, and DPP4-adipokine interactions: logical reasoning and known facts about bone related effects of DPP4 inhibitors

Article Type: Review article

Keywords: DPP4; bone; dipeptidyl peptidase 4; DPP4 substrates; adipokines; DPP4 inhibition; DPP4 inhibitors; osteoporosis

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Abstract: Dipeptidyl peptidase 4 (DPP4) is a conserved exopeptidase with an important function in protein regulation. The activity of DPP4, an enzyme which can either be anchored to the plasma membrane or circulate free in the extracellular compartment, affects the glucose metabolism, cellular signaling, migration and differentiation, oxidative stress and the immune system. DPP4 is also expressed on the surface of osteoblasts, osteoclasts and osteocytes, and was found to play a role in collagen metabolism. Many substrates of DPP4 have an established role in bone metabolism, among which are incretins, gastrointestinal peptides and neuropeptides. In general, their effects favor bone formation, but some effects are complex and have not been completely elucidated. DPP4 and some of its substrates are known to interact with adipokines, playing an essential role in the energy metabolism. The prolongation of the half-life of incretins through DPP4 inhibition led to the development of these inhibitors to improve glucose tolerance in diabetes. Current literature indicates that the inhibition of DPP4 activity might also result in a beneficial effect on the bone metabolism, but the long-term effect of DPP4 inhibition on fracture outcome has not been entirely established. Diabetic as well as postmenopausal osteoporosis is associated with an increased activity of DPP4, as well as a shift in the expression levels of DPP4 substrates, their receptors, and adipokines. The interactions between these factors and their relationship in bone metabolism is therefore an interesting field of study.

We thank you for the time invested reading this manuscript carefully and for your proposals to improve our paper. We hereby submit an adapted, more complete manuscript to Bone. Below, please find our reaction to your comments and the description of the modifications applied to the text (in italic).

Reviewer #1: I read with interest the manuscript entitled "Boning up on DPP4, DPP4 substrates, and DPP4-adipokine interactions: logical reasoning and known facts about bone related effects of DPP4 inhibitors" that try to summarize the recent findings about dpp4 actions in bone. This area of research is topical as the present and it is very important to write such review for the bone community to better understand the potential impact of this pathway in bone physiology.

The English syntax is correct although few typos have been noticed (see below). However, several limitations dampened my enthusiasm:

- A clear goal for this review should be stated. I understand totally the rationale for drafting such manuscript as it is my field of research, but I guess that the benefit of this manuscript would be better perceived by nonspecialists if a clear aim was delineated.

A sentence was added at the end of the 'Introduction' to explain the value of understanding the complex relationship between DPP4 and energy and bone metabolism.

- I acknowledge that the authors provided figure 1 and Table 1 but I found that this manuscript lack illustrations. Indeed, we do not have any idea of which tissue may produce DPP4. A table describing the expression of DPP4 in the body could be made easily by summarizing the open access data of the human protein atlas (<http://www.proteinatlas.org>). Also, the manuscript would gain if the authors produced a Figure/Table with the source of DPP4 in bone (bone cells vs. non bone-produced DPP4).

It is a challenge to provide a graphical overview of the regions of expression of DPP4 (and also the substrates). The beginning of the introduction of DPP4 was rephrased to clearly include its regions of expression, and an extra figure was added to illustrate the regions of expression of DPP4 and some of the DPP4 substrates that will be discussed later.

It is indeed useful to repeat the origin of DPP4 activity in the bone, which is soluble DPP4, DPP4 anchored to the surface of circulating cells and osteoblasts and osteoclasts. This information was added to the beginning of the second section (DPP4 and the bone). An additional table was not included as it would not provide more information.

- I would also appreciate to have in Table 1 a more profound summary of action of different DPP4 substrate than provided at the present. As an example, GIP also improves bone strength by ameliorating collagen crosslinking and the degree of bone mineralization. Although this has been reported in the manuscript it is lacking in Table 1.

A few of the effects mentioned in the manuscript were indeed not summarized in the table. The table has been completed in accordance to the text in the manuscript.

- As a rule of thumbs, it would also improve the manuscript if the authors used the same method of description for each substrate, i.e. direct effects on bone cells, effects in animal models (KO when exist and then effects of agonist/antagonist) and finally clinical data.

In our initial draft, we already tried to include exactly this order to describe each individual substrate. However, due to the nature of the text, description of the effects was rearranged to improve readability. The descriptions for each of the substrates was reread and some paragraph rearrangements were made to make this method of description more clear. Nevertheless, the lack of clinical studies evaluating the effects of some substrates (SDF-1 α , NPY, PYY ...) makes it difficult to maintain the same method of description for each substrate.

- The discussion section is purely speculative and should be discarded.

The discussion was renamed to "summarizing and concluding remarks" and substantially shortened.

Minor comments:

- Abstract: replace "cellular membrane" by "plasma membrane"

Corrected

- Last sentence of abstract is difficult to read and should be rewritten

Sentence was rewritten

- GIP stands for "Glucose-dependent insulintropic polypeptide" and not "glucose-dependent insulinogenic polypeptide"

Corrected

- Page 6, line 33: a ref should be added to support the elevated GIP after a meal

Reference added

- Page 7, line 18: ref 37 investigated the effects of GIP analogue in type 1 diabetic mice, not rats

Corrected

- Page 8, line 29: ref 20 did not investigate the presence of a second GLP1r in osteoclast. The sentence should be corrected

Rephrased to two sentences

- Page 8/9: the role of GLP1 in bone quality is not discussed whilst such information exist in the literature (at least in Glp1r KO and type 1 diabetic mice)

Elaborated further on the 2015 Mansur reference and added another 2013 article from the Mabileau group

- Page 10, line 26: remove "exclusively" or add a reference, as ref 44 and 64 did not investigate whether GLP2r is only present in osteoblasts.

The word "exclusively" was removed, and a sentence was added regarding the presence of the GLP-2 receptor in early osteoblastic cell lines (with reference)

- Page 15, line 20: the comma between 93 and 81 should be superscript

Corrected

- Page 16: data on IGF binding protein should be added or at least discussed if no data exists

This information was omitted as we considered it less relevant. No data was found regarding the direct effect of DPP4 on IGF-binding proteins. However, considering truncation by DPP4 causes truncated IGF-1 to bind more readily to IGFBP3, we do agree it could hold meaning and therefore have now included this at the end of the paragraph about experimental studies regarding IGF-1.

- Leptin, insulin, adiponectin, ghrelin, amylin and preptin are also DPP4 substrates; as such the dichotomy of paragraph 4 is odd and should be discarded

According to our literature research, none of these peptides are known physiological substrates of DPP4. Leptin has a pharmacological DPP4 truncation site (penultimate proline), but to the best of our knowledge no relationship has been drawn between active leptin and

DPP4 activity. The peptide ghrelin was even found to correlate with DPP4 activity. As these peptides are involved in energy metabolism as well as the bone, but not recognized as physiological DPP4 substrates (although some have another relationship to DPP4), this distinction was made.

- The reference section should be checked carefully as many of the reference do not comply with Bone style

The updated Bone reference style was downloaded, applied and verified.

The authors are thankful to the reviewer for his constructive remarks which undoubtedly have enhanced the quality of our manuscript.

***Highlights (for review)**

- DPP4 and its substrates play an important role in energy metabolism and the bone
- Expression of DPP4 and substrates can be altered through (bone) pathology
- Nutritional peptides that closely interact with DPP4 also affect the bone
- Inhibition of DPP4 activity may result in a beneficial effect on the bone

**Boning up on DPP4, DPP4 substrates, and DPP4-
adipokine interactions: logical reasoning and known
facts about bone related effects of DPP4 inhibitors**

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The authors have no conflicts of interest to disclose.
No supplemental data has been included with the submission.

Abstract

Dipeptidyl peptidase 4 (DPP4) is a conserved exopeptidase with an important function in protein regulation. The activity of DPP4, an enzyme which can either be anchored to the plasma membrane or circulate free in the extracellular compartment, affects the glucose metabolism, cellular signaling, migration and differentiation, oxidative stress and the immune system. DPP4 is also expressed on the surface of osteoblasts, osteoclasts and osteocytes, and was found to play a role in collagen metabolism. Many substrates of DPP4 have an established role in bone metabolism, among which are incretins, gastrointestinal peptides and neuropeptides. In general, their effects favor bone formation, but some effects are complex and have not been completely elucidated. DPP4 and some of its substrates are known to interact with adipokines, playing an essential role in the energy metabolism. The prolongation of the half-life of incretins through DPP4 inhibition led to the development of these inhibitors to improve glucose tolerance in diabetes. Current literature indicates that the inhibition of DPP4 activity might also result in a beneficial effect on the bone metabolism, but the long-term effect of DPP4 inhibition on fracture outcome has not been entirely established. As ~~diabetic~~ diabetic as well as postmenopausal osteoporosis is associated with an increased activity of DPP4, as well as a shift in the expression levels of DPP4 substrates, their receptors, ~~and as well as~~ adipokines, ~~the~~ interactions between these factors and their relationship in bone metabolism is therefore an interesting field of study.

Keywords: DPP4, bone, dipeptidyl peptidase 4, DPP4 substrates, adipokines, DPP4 inhibition, DPP4 inhibitors, osteoporosis

Abbreviations: ALP: alkaline phosphatase, APN(R): adiponectin (receptor), BMD: bone mineral density, Coll1: collagen type 1, CXCR4: C-X-C chemokine receptor 4, DPP4: dipeptidyl peptidase 4 (CD26: cluster of differentiation 26), GLP-1/2: glucagon-like peptide 1/2, GIP: glucose-dependent insulinotropic peptide, IGF-1: insulin growth factor 1, LP(R): leptin (receptor), MMP: matrix metalloproteinase, NK-1R: neurokinin 1 receptor, NPY: neuropeptide Y, OCN: osteocalcin, OPG: osteoprotegerin, PACAP: pituitary adenylate-cyclase activating protein, PTH: parathyroid hormone, PYY: peptide YY, RANK(L): Receptor activator of nuclear factor kappa-B (ligand), SDF-1 α : stromal-derived factor 1 α , SP: substance P, VIP: vasoactive intestinal peptide.

1. Introduction

Dipeptidyl peptidase 4 (DPP4, or CD26) is a highly conserved and ubiquitously expressed dimeric transmembrane exopeptidase found mostly on the surface of endothelial[1] and epithelial cells[2], but also on immune cells[3]. Highest expression levels in humans have been reported to occur on the surface of epithelial cells at the brush border of the proximal convoluted tubules in the kidney and of the small and large intestine. It can also be found on the surface of glandular genital cells, liver hepatocytes and others. However, DPP4 also exists in a soluble form in the circulation and on the surface of activated leukocytes (T-, B- and natural killer cells)[4]. DPP4 specifically cleaves off X-Pro and X-Ala dipeptides from polypeptide substrates[5]. The activity of this oligopeptidase exerts major influence on the glucose metabolism, signal transduction, cell migration, cell differentiation and the immune system by deactivating or modulating the activity of its substrates, and by interacting with other proteins[4]. Figure 1 gives an overview of the regions of expression of DPP4 and some of its substrates which will be discussed later in this review, as they are of importance for bone metabolism.

Figure 1. Important regions of expression of DPP4 and DPP4 substrates (●) and their receptors (○) that play a role in bone metabolism. DPP4 and some other peptides are also present in the circulation.

Although DPP4 exerts a seemingly important regulatory role, DPP4 knockout mice are phenotypically normal, have an improved glucose metabolism and show more physical activity. However, at a later age, they display a decreased number of T-cells, B-cells and NK-cells and an increased number of regulatory T-cells[6]. The regulation of DPP4 activity is not only dependent on DPP4 expression itself but also on the availability of its substrates[4]. Various

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9 pathophysiological conditions have been associated with either an increase or a decrease of
10 DPP4 expression. DPP4 release was shown to correlate with adipocyte size, obesity[7] and
11 hyperglycemia[8], suggesting that DPP4 is strongly involved in the relationship between these
12 factors[9]. In healthy subjects, an oral glucose load also goes along with a transient increase of
13 serum DPP4 levels, associated with an increased activity of adiponectin and hepatic
14 enzymes[10]. In case of ischemic damage, a quick up-regulation of soluble DPP4 is observed in
15 the serum[11]. Various cancers as well as other pathologies have been associated with increases
16 in DPP4 expression[12]. The presence of soluble DPP4 as well as DPP4 expression on the
17 surface of T-lymphocytes is elevated in asthmatic patients[13]. On the other hand, some
18 pathologies are linked to a decreased DPP4 activity[14, 15]. DPP4 inhibitors were developed to
19 improve the glucose tolerance in type 2 diabetic patients by prolonging the half-life of the
20 incretins glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide 1
21 (GLP-1), which promote the secretion of insulin by β -pancreatic cells[4]. In the last decade,
22 DPP4 inhibitors have been developed and approved for use, of which vildagliptin, sitagliptin,
23 linagliptin and saxagliptin are the most popular ones. Many others are still in development.

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39 Some pathologies of the bone metabolism have also been associated with an alteration of DPP4
40 activity. In this article, a summary is given of current knowledge regarding DPP4 and the bone,
41 of important DPP4 substrates that have an established role in the bone metabolism, and of other
42 peptides related to DPP4 and the energy metabolism, that also have known implications on the
43 bone metabolism. The interactions between these factors are important elements to understand
44 the relationship between energy and bone metabolism.
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2. DPP4 and the bone

Throughout the years, literature not only has shown that DPP4 is important for protein regulation in the context of energy metabolism, but also that DPP4 activity has direct as well as indirect implications on bone metabolism. In the bone, DPP4 is present in the circulation in soluble form, on the surface of circulating cells and on the surface of osteoblasts[16] and osteoclasts[17].

Degradation of collagen occurs through a complex interaction of various proteins among which matrix metalloproteinases (MMP), prolidase and DPP4 are key players. Fragments of alpha collagen chains processed by proteases contain glycine-proline sequences, which are recognized by DPP4 and broken down to small dipeptides[18]. But this is not the only contribution of DPP4

in bone metabolism. ~~DPP4 is also expressed on the surface of osteoblasts[17] and osteoclasts[18].~~ Many substrates of DPP4 as well as their receptors are expressed in

osteoblasts[19-22], osteoclasts[20, 21, 23] and osteocytes[24, 25] (figure 24). Furthermore,

DPP4 substrates are present in the circulation, and some are locally released by nerve fibers.

DPP4 activity is correlated with hyperglycemia in diabetes and associated to osteoporotic bone loss[8] and osteoporosis in general[26], indicating that the regulation of DPP4, if not directly involved in the pathophysiology of these bone diseases, is affected by their development. The effect of some DPP4 substrates on the bone has clearly been established in experimental studies[20, 27-29]. Furthermore, some meta-analyses of clinical studies regarding the effects of DPP4 inhibitors and GLP-1 based therapies on the bone have been published[30]. Nevertheless, the mechanisms through which DPP4 activity affects the bone metabolism have never been completely elucidated. Therefore it is of utmost relevance to summarize bone related effects of

DPP4 substrates together with the known or (by logical reasoning) expected effects of DPP4 and
DPP4 inhibition ~~on these effects~~.

3. DPP4 substrates and the bone

3.1. Animal studies provide evidence that GIP increases bone formation and reduces bone resorption

Glucose-dependent insulinotropic peptide (GIP) is mainly originating from the proximal small intestine in response to nutrient ingestion[31]. Circulating GIP-levels increase up to five-fold after a meal[32]. GIP is degraded by DPP4 with a relatively short half-life of about four minutes in human serum[33].

In the bone, the receptor for GIP (GIPR) was found on the cellular membrane of osteoblast cell lines and bone marrow stem cells[19]. GIP binding to GIPR results in an increased proliferation of SAOS-2-cells (human osteosarcoma cells) and intracellular Ca^{2+} and cAMP concentrations, coupled with an increased alkaline phosphatase (ALP) activity and collagen type 1 (Coll1) mRNA expression[24]. In mice osteoblast cell culture (MC3T3-E1 cell line), GIP was found to improve collagen maturation by crosslinking as well as by affecting fibril diameter[34]. GIPR expression was also observed on osteoclasts, and binding of GIP to its receptor inhibits bone resorption[23]. Intravenous GIP administration was shown to prevent bone loss in ovariectomized rats[23] and to increase bone mineral density (BMD)[27]. Even in control rats, GIPR expression is correlated with BMD[23]. GIPR was also found on the surface of osteocytes in rat bone[24]. The variation of GIPR expression observed when comparing different osteoblastic cell lines suggests a variable expression during osteoblastic differentiation[35].

In experimental animals, ~~t~~The expression of functional GIPRs has also been shown to decrease with age, an effect which can be compensated by GIP administration[36]. A significant increase of bone strength was observed in the cortical bone of rats undergoing treatment with an N-terminally modified DPP4-resistant GIP, associated with increased mineralization and collagen maturity in the bone matrix[37]. A similar effect was observed in type 1 diabetic ~~mice~~^{rats} undergoing the same treatment, countering rapid bone loss induced by diabetes[38]. In GIPR knockout mice, a reduced BMD was observed[27], as well as an altered bone microarchitecture and bone turnover[39]. Cortical osteopenia in these knockouts results in a significant reduction in ultimate load and stiffness of the femoral bone, suggesting a reduced bone strength and quality[40]. Although cortical bone was generally found deteriorated in GIPR knockout mice, one study reported an increase of trabecular bone volume and trabecular number[41]. This could be caused by a change of the adipokine expression profile in GIPR knockouts, which could affect cortical and trabecular bone differently[42]. Furthermore, these knockout mice show reductions in parameters of bone formation as well as increases in plasma calcium levels after meal ingestion, suggesting GIP to serve as an important link between feeding and calcium deposition in the bone[43].

In (healthy) humans, GIP infusion was found to result in reduced bone resorption markers on short term in healthy humans[44], but the long-term effect of GIP administration on bone turnover has not been studied in a clinical setting[45].

From animal studies, it can be concluded with overwhelming evidence that GIP has anabolic effects on the bone, which in turn allows to suggest that DPP4-inhibition would positively affect the bone in conditions of reduced bone formation or bone loss. It needs to be mentioned however, that clear-cut long-term evidence for such an effect in humans is still lacking.

3.2. GLP-1 has an anabolic effect on the bone in cell culture and animal studies

Glucagon-like peptide 1 (GLP-1) is an incretin hormone originating from the distal small intestine in response to feeding and central signaling molecules, acetylcholine and gastrin-releasing peptide[19]. The plasma concentration of GLP-1 is susceptible to changes of the intestinal environment[46], and is stimulated through peptides like truncated peptide YY[47] and the uncarboxylated form of osteocalcin (OCN)[48]. As measured in the capillaries of the L cells of the porcine intestine, active GLP-1 gets truncated by DPP4 with a half-life of one to two minutes, thereby losing its insulinotropic effect[49].

In vitro, the GLP-1 receptor was found on the surface of bone marrow cells, primary osteoclasts and osteoblasts, of a late osteocytic cell line[50]. Two types of GLP-1 receptors can be found on active osteoblasts ~~whilst only one type is seen in osteoclasts~~[20]. GLP-1 was found to exert an insulin- and PTH-independent anabolic action on bone in control as well as diabetic rats[29]. The same authors reported that GLP-1, administered to (mouse) osteoblastic MC3T3-E1 cells, increased the expression of OCN and osteoprotegerin (OPG), decreased the expression of Runt-related transcription factor 2 (Runx2) and promoted proliferation/inhibited apoptosis[20].

In line herewith, GLP-1 receptor agonists were shown to reverse hyperlipidemic osteopenia in rats through the increased expression of OCN and OPG, resulting in increased bone mass and normalization of bone remodeling[51].

In GLP-1R knockouts (Glp-1r^{-/-}) it was evidenced that endogenous GLP-1 receptor signaling, through a calcitonin-dependent pathway, has an essential role in the postprandial suppression of bone resorption[40]. GLP-1R was also found on osteocytes, where it was shown to co-localize

with sclerostin and to reduce the expression of this protein which is known to inhibit osteoblastic activity and stimulate catabolic actions on bone[25].

Bone anabolic action of GLP-1 is furthermore confirmed by animal studies investigating the effect of GLP-1 on BMD, bone strength and bone architecture. GLP-1 administration was found to result in an improvement of bone architecture in streptozotocin-induced diabetic rats and insulin-resistant rats[29, 52]. Furthermore, bone strength could be ~~maintained prevented~~ with the GLP-1R agonist liraglutide in type 1 diabetic mice through preventing the deterioration of the quality of the bone matrix[38]. The reduction of bone quality and strength in GLP-1R knockout mice confirms the need of the GLP-1R for optimal bone quality through its beneficial effect on intrinsic bone matrix properties, associated with improved maturation of collagen[53]. Also in aged ovariectomized rats, GLP-1R agonists were shown to prevent osteopenia through the promotion of bone formation as well as suppression of bone resorption[54, 55]. Similar to GIPR, hyperglycemia is associated with a down-regulation of GLP-1R, resulting in a reduced efficiency of GLP-1 agonists as a treatment for diabetics[56]. Furthermore, GLP-1 induces an increase of sodium excretion, which is associated with trabecular bone loss through increased osteoclast activity and modulation of OCN[57].

A study conducted in obese women during long-term weight loss concluded that the GLP-1R agonist liraglutide is able to prevent bone loss and ~~even to~~ increase bone formation[58]. Recent clinical meta-analyses of fracture outcome in type 2 diabetic patients however, suggested a neutral effect of GLP-1 receptor agonists on fracture risk[59-61].

~~Similar to GIPR, hyperglycemia is associated with a down-regulation of GLP-1R, resulting in a reduced efficiency of GLP-1 agonists as a treatment for diabetics~~[57]. Furthermore, ~~GLP-1~~

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~~induces an increase of sodium excretion, which is associated with trabecular bone loss through increased osteoclast activity and modulation of OCN[58].~~

In conclusion, as for GIP, the anabolic effect of GLP-1 on the bone has been shown in various experimental studies comprising healthy as well as diabetic animals, again pointing to a protective effect of DPP4 inhibition against bone loss. ~~The Recent clinical meta-analyses of fracture outcome in type 2 diabetic patients however, suggested a neutral effect of GLP-1 receptor agonists on fracture risk[59-61].~~ anabolic effect of GLP-1 however, so far has not been confirmed in clinical studies.

3.3. GLP-2 effectuates postprandial reduction and inhibits increased nocturnal bone resorption, as evidenced by both animal and clinical studies

Glucagon-like peptide 2 (GLP-2) is a gastrointestinal peptide which is released from the distal small intestine in response to nutrient ingestion and other specific non-nutrient stimulators. In addition, it is produced in the central nervous system. In the intestine, GLP-2 is co-secreted with GLP-1[62]. GLP-2 is deactivated by circulating DPP4 with a half-life of five to seven minutes in human serum[63]. Like GLP-1, GLP-2 levels are modulated by the intestinal environment[46]. Expression of GLP-2 and GLP-2R is variable according to the developmental stage in intestinal tissues[64]. The GLP-2 receptor (GLP-2R) is mostly expressed in the stomach and bowel, but also in heart, kidney, liver, brain and ~~the~~ bone[65]. Experiments with lean as well as obese mice conclude that the administration of GLP-2 results in a reduction of food intake[66].

In the bone, GLP-2R ~~can be~~ is exclusively found on osteoclasts, and the binding of GLP-2 on its receptor is associated with a reduction of bone resorption, independent of changes in bone

formation[45, 67]. The in vitro expression of GLP-2R was shown in early -osteoblastic cell lines MG-63 and TE-85, but not in more differentiated osteoblasts[35].

GLP-2 in the context of the bone was mostly studied in human subjects. GLP-2, like GIP and GLP-1, is responsible for the attenuation of postprandial bone resorption~~[40, 45]~~[45]. Various other clinical studies have confirmed the effect of GLP-2 as inhibitor of bone resorption by investigating serum levels of bone resorption markers[28, 68]. GLP-2 administration was found to result in the inhibition of the nocturnal rise of bone resorption in healthy postmenopausal women, also after parenteral and subcutaneous administration[69]. In another clinical study conducted with gastric bypass surgery patients, the severely increased postprandial GLP-2 response observed after the procedure was associated with an increased bone turnover[70]. The increase of BMD after GLP-2 administration is generally found to be dose-dependent[28] and more effective after long term exposure[71].

Overall, results provide evidence for an anti-resorptive effect of GLP-2, indirectly implying a beneficial effect of DPP4 inhibition on the bone.

3.4. The SDF-1 α /CXCR4-axis is a strong chemotactic for active bone cells as shown in vivo in mice and in vitro using human progenitor cells

Stromal derived factor 1 alpha (SDF-1 α) is ubiquitously expressed and is the most efficiently deactivated substrate of DPP4, with a half-life of less than one minute as measured in an *in vitro* assay using DPP4 from human seminal plasma[72]. It can be found in the bone in mature osteoblasts, osteocytes, osteoblast progenitor cells and the endothelial lining cells[73]. Its

receptor CXCR4 can be found anchored to the surface of osteoclast progenitor cells and osteoclasts[73].

In mice and *in vitro* using human progenitor cells, the SDF-1 α /CXCR4 chemotactic signal was found to play a major autocrine role in the regulation of osteoblast activity as well as transdifferentiation of progenitor cells and their migration to sites of bone formation[74, 75]. SDF-1 α also increases osteoblast proliferation and collagen type 1 expression *in vitro* in cultured osteoblasts from osteoarthritis patients[76]. *In vitro*, it also promotes early stages of human osteoclast differentiation resulting in increased cell numbers and increased resorption activity, as well as the prevention of osteoclast apoptosis[73].

Furthermore, animal studies revealed that the SDF-1 α /CXCR4-axis plays a role in lipopolysaccharide-induced inflammatory bone resorption through the upregulation of CXCR4 in pre-osteoclasts[77] and that the SDF-1 α and CXCR4 expression increases in response to mechanical stimulation. The above mentioned studies all point to an important role for the SDF-1 α /CXCR4-axis in bone cell recruitment. One single animal study, using a CXCR4 receptor antagonist reported a significantly attenuated load-induced bone formation[78].

Till today no clinical studies assessing the effect of exogenous administration of SDF-1 α and/or CXCR4 antagonists on bone metabolism or bone mineral density have been conducted.

To which extent DPP4 inhibition in the presence of intact SDF-1 α activity may affect bone metabolism, currently is still prone to speculation.

3.5. NPY inhibits osteoblast activity through the central and peripheral nervous system as evidenced by experimental animal and in vitro studies

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9 Neuropeptide Y (NPY) is a neurotransmitter expressed in many tissues and considered to be the
10 most abundant peptide in the heart as well as in the central and peripheral nervous system[79]. It
11 plays an important role in inflammation and oxidative stress[80]. Human NPY is truncated by
12 DPP4 with a variable half-life of two to three minutes depending on the region of expression[81].
13 NPY binds to the G protein linked Y-receptors Y1, Y2, and Y5[79]. These receptors have a
14 variable distribution across different cell types and mediate different functions. After truncation
15 of NPY(1-36) to NPY(3-36) by DPP4, the protein loses the ability to bind to the Y1 receptor but
16 remains capable of exerting all other non-Y1 mediated functions until it gets further deactivated
17 by other peptidases[82]. The Y2 receptor is responsible for neuro-inhibitory effects of NPY in
18 the central and peripheral nervous system, and also exerts an anti-obesogenic role in the
19 brain[83]. In the hypothalamus, NPY co-regulates food uptake by binding on Y1 and Y5
20 receptors to increase appetite[84]. Increased hypothalamic NPY expression propagates
21 orexigenic signals via Y1 and Y5 receptors, inducing skeletal responses. The skeleton is widely
22 innervated and NPY was shown to be expressed by skeletal nerve fibers in the bone to exert
23 paracrine biological effects[85]. The interplay between the hypothalamus and the bone is shown
24 by the hypothalamic control of bone formation, leptin stimulation of hypothalamic nuclei and the
25 presence of specific peptide receptors on osteoblasts and osteoclasts. -Menopause also illustrates
26 this interplay, involving a decreased sensitivity to leptin due to estrogen deficiency and an
27 increased NPY expression in the brain, affecting the energy metabolism[83, 86, 87]. The Y1
28 receptor was also found to be variable throughout the menstrual cycle[88, 89].

29 In the bone, NPY is expressed in osteoblasts, osteocytes, osteoclasts and chondrocytes[21]. In
30 isolated cell populations derived from neonatal rat calvariae, the osteocyte fraction showed an
31 increased NPY expression compared to osteoblasts[90]. The effect of NPY on the bone has not

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9 been evaluated in clinical studies, so the below summary is limited to results obtained by *in vitro*
10 and experimental animal studies.

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13 Animal studies revealed that NPY produced by peripheral sources, most notably during stress,
14 can signal directly to Y1 receptors in osteoblasts and Y1 and Y2 receptors in adipocytes,
15 resulting in a reduction of the response to mechanical load induced bone formation and a
16 stimulation of lipid accumulation[83]. Indeed, NPY inhibits osteoblast activity in primary cell
17 cultures of rat calvariae through the Y1 receptor[91]. Mice lacking NPY have a significantly
18 increased bone mass in association with an enhanced osteoblast activity, an effect found to be
19 dependent on the osteoblastic Y1 receptor and independent of the hypothalamic Y1 receptor.
20
21 Overexpression of NPY in the hypothalamus of these as well as wild-type mice results in a
22 reduction in bone mass despite developing an obese phenotype. This induced loss of bone mass
23 is consistent with mice models known to mimic the central effects of fasting, which also show
24 increased hypothalamic NPY levels. Osteoblast-specific Y1 knockout mice have an increased
25 bone mass and severely altered glucose homeostasis with an increased serum glucose and a
26 decreased glucose-mediated insulin response[84, 92]. Finally, administration of NPY to rat
27 calvaria-derived osteoblast cultures results in a reduced expression of markers of osteoblast
28 differentiation[91]. In conclusion, the above data point to direct NPY binding to osteoblastic Y1
29 receptors thereby decreasing osteoblastic activity. Nevertheless, increased NPY activity has also
30 been related to increased bone mass through currently ~~unnon~~-explained mechanisms[90].

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33 NPY was also shown to exhibit an indirect anti-osteogenic effect on trabecular (not on cortical)
34 bone through hypothalamic NPY-expressing neurons. These effects of NPY on the hypothalamus
35 through Y2 receptor signaling were found to be counteracted by effects of leptin binding[83].
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37 Thus Y1 receptor pathways act powerfully to inhibit bone formation in osteoblasts directly,

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9 while both Y1 and Y2 receptor pathways may indirectly affect bone metabolism through
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11 hypothalamic pathways[85, 93, 94].
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13 Because of the direct osteoblast inhibitory action of Y1 receptor binding, Y1 receptor antagonists
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15 were proposed as potential drugs to prevent bone loss[95]. With regard to the latter it needs to be
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17 noted however, that chronically elevated levels of NPY lead to [a](#) transient down-regulation of the
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19 Y1 as well as Y2 receptor expression.
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21 With respect to bone resorption, NPY inhibits the transcriptional activity of the RANKL
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23 promotor in osteoprogenitor cells and enhances OPG expression in osteoblasts at early stages of
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25 differentiation, and thus can be considered an inhibitor of bone resorption. This effect of NPY on
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27 OPG seemed to be unrelated to Y2 receptor activation[96]. Recently, NPY was shown to protect
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29 against excessive stress-induced bone loss through Y2 receptor-mediated modulation of central
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31 and peripheral neurons[97].
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33 In conclusion, evidence has been provided for a linkage between reduced bone formation and
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35 central Y2 receptor and osteoblastic Y1 receptor signaling of NPY. In line herewith, loss of NPY
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37 expression or receptor signaling has been shown to increase osteoblast activity and bone mass.
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39 Moreover NPY regulates bone turnover as a critical integrator of bone homeostatic signals,
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41 increasing bone formation in the condition of excess energy storage and low hypothalamic NPY
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43 expression levels, and reducing bone formation under conditions of low energy and high
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45 hypothalamic NPY expression levels[93]. In light of the fact that DPP4 is only able to inhibit Y1
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47 signaling, evaluating the net effect of DPP4 inhibition on NPY-related bone effects is
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49 challenging.
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3.6. PYY levels are ~~inversely negatively~~ correlated with osteoblastic activity in animals and humans

Peptide YY (PYY) is a gastrointestinal hormone mostly secreted postprandially in the ileum and colon together with GLP-1, to suppress appetite, increase satiety and limit meal size[98, 99]. Post-prandial PYY release is stimulated by nutrients in the intestine, and regulated by cholecystokinin and vasoactive intestinal peptide (VIP). PYY ~~activityactivity~~ is inhibited by a fasting state[84, 99]. Reduced PYY levels are typically ly seen in obese subjects, and administration of PYY results in a reduced appetite and decreased food intake in obese adults[84, 100]. Human PYY is truncated by DPP4 with a half-life of eight to twelve minutes according to the region of expression[99]. PYY shares the same receptors with NPY and also loses Y1 binding capacity after processing by DPP4[99].

PYY exerts more or less the same effect on the bone metabolism through the osteoblast Y1 receptor and the hypothalamic Y2 receptor. PYY, however, is not secreted in the bone itself. Like NPY knockout animals, PYY knockouts have an increased bone mass and an increased osteoblastic activity[98]. Overexpression of PYY results in an increased osteoclast surface in the femoral metaphysis, despite the lack of PYY receptor expression in osteoclasts[98]. In contrast, earlier studies concluded that PYY administration resulted in an increase of BMD and PYY knockout mice had a reduced trabecular bone volume[101]. However, this could be attributed to the difficulty of producing mice with genetically altered levels of PYY, which can have lethal consequences during embryonic development in case of overexpression[102].

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In premenopausal exercising women, PYY levels are elevated, and in combination with suppressed estrogen concentrations, could directly contribute to low BMD and amenorrhea[103].

In anorexic adolescents, increased PYY levels are also related to a reduction of BMD[100].

~~In contrast, earlier studies concluded that PYY administration resulted in an increase of BMD and PYY knockout mice had a reduced trabecular bone volume[102]. However, this could be attributed to the difficulty of producing mice with genetically altered levels of PYY, which can have lethal consequences during embryonic development in case of overexpression[103].~~ The

NPY and PYY network is complex and works in close association with noradrenaline circuits and feeding peptides[84]. This makes it a challenge to assess the exact interaction between these peptides and their effect(s) on bone in clinical studies, as well as to evaluate the effect of DPP4 inhibition on this interaction and on the bone, given the fact that DPP4 selectively inhibits Y1 signaling.

3.7. IGF-1 promotes osteoblast proliferation and reduces bone resorption, as evidenced by animal studies and suggested by human studies

Insulin growth factor 1 (IGF-1) is produced primarily by the liver and is stimulated by insulin and growth hormone. Malnutrition or disturbances in the growth hormone stimulatory pathway delay the production of IGF-1. ~~In rats,~~ IGF-1 is deactivated by DPP4 in the circulation with a half-life of 10 minutes in rat serum[104]. In the bone, IGF-1 and its receptor are expressed by osteoblasts and osteocytes[105].

In mice, the binding of IGF-1 to its receptor on osteoblasts stimulates the expression of ALP and OCN[106] and IGF-1 also stimulates the proliferation and chemotaxis of osteoblasts[107]. It is

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noteworthy that, half of the basal bone cell proliferation can be blocked by inhibiting the actions of endogenously produced IGF-1[108]. IGF-1 receptor expression decreases whereas insulin receptor expression increases during osteoblast differentiation[109]. IGF-1 was also shown to be an important mediator for the actions of PTH on the bone[110]. PTH, a hormone which also exerts a major influence on bone turnover and which is modulated through various pathologies, in itself is an inhibitor of DPP4 activity[111]. Furthermore, in mice, IGF-1 indirectly reduces bone resorption through an increase of OPG and a decrease of RANKL expression[106]. The truncation of IGF-1 by DPP4 makes it less inclined to activate IGF-1R, but more prone to bind to IGF-binding protein 3 (IGFBP3)[112]. It is not clear how this could also affect the bone, but it has been shown that a co-administration of IGF-1 and IGFBP3 resulted in an increased concentration of IGF-1 in the bone matrix, and a stimulation of bone formation in aged rats[113]. A knockout in the IGF-1R results in severe growth retardation and a reduced osteoblastic activity.

Whereas the expression of IGF-1 is reduced in a state of poor nutrition, the long term feeding with protein supplements causes an increase of IGF-1 associated with an increase of hip BMD in adolescent girls as well as in older patients with previous hip fracture[114, 115]. ~~A knockout in the IGF-1 receptor on the other hand results in severe growth retardation and a reduced osteoblastic activity.~~ In general, serum IGF-1 levels are directly correlated with BMD and inversely correlated with the risk of fractures in postmenopausal non-diabetic women and in dialysis patients which makes that it might be considered an interesting biomarker[116]. In conclusion, IGF-1 is a DPP4 substrate that most probably has a positive effect on the bone, which again underscores the potential of DPP4 inhibition to exert positive effects on bone health.

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9 *3.8. Cell culture experiments reveal that VIP, PACAP and SP stimulate osteoblasts and with*
10 *exception of SP decrease osteoclastic activity*
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15 VIP, pituitary adenylate cyclase-activating peptide (PACAP) and substance P (SP) are expressed
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17 by peptidergic nerve fibers in the bone, and are another example of hypothalamic control of bone
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19 formation[117]. In human plasma they are truncated by DPP4 with a half-life of two, five and ten
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21 minutes, respectively[118-120]. VIP binds on VIP receptor types 1 and 2 (VPAC1 and VPAC2),
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23 PACAP binds the VIP receptors and an additional PAC1 receptor, and SP binds the neurokinin 1
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25 receptor (NK-1R). Osteoblasts express the receptors VPAC1, VPAC2 and NK-1R[121, 122]
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27 whereas osteoclasts present VPAC1, PAC1 and NK-1R on their surface[123]. Neuropeptides
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29 VIP and SP exert their paracrine effects in close relation with leptin stimulation of hypothalamic
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31 nuclei mediated by the sympathetic nervous system and inhibitory and adrenergic receptors on
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33 osteoblasts[121]. Results concerning effects of VIP, PACAP and SP on the bone are mainly
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35 obtained from cell culture experiments. VIP, without affecting cell proliferation, stimulates
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37 osteoblastic ALP synthesis and bone nodule formation[122]. Binding of VIP as well as PACAP
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39 to receptors on osteoclasts is linked to a transient inhibition of osteoclastic activity[124]. In
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41 addition, the VIP/PACAP receptors in osteoblasts are coupled to a delay in the stimulation of
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43 osteoclastic resorption[125]. VIP also has an inhibitory effect on osteoclast recruitment, due to
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45 upregulation of OPG in osteoblasts and down-regulation of RANK in osteoclasts[126]. Adding
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47 VIP to bone marrow cultures was found to result in a decreased formation of osteoclasts and an
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49 increase of osteoblast activity[126]. In rat osteoblast cultures, PACAP signaling was shown to
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51 enhance osteogenesis increasing the expression of Coll1, osterix and ALP[127]. SP also
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stimulates osteoblastic bone formation[128] and additionally plays a role in osteoblastic differentiation[129].

On the other hand, SP was also found to potentiate the osteoclastogenic response to RANKL by increasing RANK expression and the size of resorption pits *in vivo*. Also, in some models of vertebral injury, the increased presence of SP-immunoreactive fibers was associated with osteoporotic phenotypes[122]. Whereas VIP and PACAP could be expected to have positive effects on the bone *in vivo*, the potential effect of SP on bone is less clear. The effects of these peptides established *in vitro* have not yet been studied in the clinical setting. VIP and PACAP could be added to the list of DPP4 substrates that with great probability are able to exert positive effects on the bone *in vivo*. The effect of SP is less clear and adds up ambiguity to the net effect of DPP4 inhibitors on bone metabolism.

4. DPP4, energy metabolism and the bone

There is a complex interplay between nutrition and the bone metabolism. Diurnal regulation of bone turnover is strongly affected by feeding and fasting. These interactions of energy homeostasis involve the previously mentioned gastrointestinal peptides, but also other peptide molecules and adipokines known to play a role in the regulation of glucose metabolism, hunger and satiety. Some of these peptides also have an established role in bone homeostasis and are associated with DPP4.

4.1. Leptin

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9 Leptin (LP) is predominantly secreted by adipocytes and is known as the hormone that induces
10 satiety. It has regulatory effects on hematopoiesis, blood pressure, T-lymphocyte function,
11 reproductive function and also has a role in bone metabolism[130]. Although leptin is not
12 recognized as an important DPP4 substrate in literature, it has a putative DPP4 truncation
13 site[131]. Furthermore, a strong association was found between the secretion of leptin and DPP4
14 by adipocytes[9, 132]. Leptin is released in proportion to fat mass and acts as a marker of energy
15 storage levels. In general, leptin concentrations in the plasma are proportional with body weight
16 and are increased in obesity. Lack of the leptin receptor signal in the hypothalamus results in an
17 obese phenotype in a permanent state of hunger, associated with an increased NPY
18 expression[133]. The leptin receptor is also expressed in osteoblasts, where leptin binding
19 promotes their proliferation and differentiation[134]. Furthermore, it reduces the production of
20 RANKL[134] and increases OPG expression, thereby reducing osteoclastogenesis[135]. *In vivo*,
21 leptin deficiency alters bone density in a dual way as it increases trabecular bone volume, but
22 reduces cortical bone volume[136], linear bone growth and growth plate thickness[134]. In
23 contrast with the above has leptin signaling also been reported to reduce trabecular bone
24 mass[137]. Anyhow, the effect of leptin on the bone is complex since it was also found to
25 perform strong inhibitory effects on bone formation through the central nervous system, and to
26 inhibit bone resorption through the modulation of RANKL[136, 138, 139]. Furthermore, leptin
27 indirectly affects the bone metabolism by increasing estradiol and IGF-1[140]. As previously
28 mentioned, leptin also regulates cortical and trabecular bone mass through NPY[97]. The
29 inhibition of insulin secretion was also recognized as an indirect downstream effect of leptin
30 signaling through the decrease of OCN activity[141]. Circulating leptin concentrations were
31 shown to be inversely related to bone resorption in postmenopausal women[142]. Resistance to
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leptin increases both with age and body mass index, making it unsuitable as a treatment, except in specific cases[133, 143]. The precise role of leptin in the bone needs still to be defined, but the pathways established so far clearly illustrate its complexity[144]. It is difficult to speculate about the exact effects of DPP4 activity and inhibition on the half-life and expression of leptin, which could ultimately affect the bone.

4.2. *Adiponectin*

Adiponectin, another peptide specifically secreted by adipocytes, is inversely correlated with body fat percentage in adults, with severely reduced circulating levels in obesity and diabetes[145]. The release of adiponectin is stimulated by GLP-1[146]. Adiponectin was found to increase insulin sensitivity and play an important role in the control of energy metabolism. The adiponectin receptor is found on osteoblasts, which also secrete adiponectin themselves, and osteoclasts. Adiponectin regulates the bone metabolism via central and peripheral mechanisms[147]. It stimulates osteoblast activity and suppresses osteoclastic bone resorption[148]. It also inhibits the differentiation of pre-osteoclasts[149] and promotes the differentiation of osteoblasts[147]. Nevertheless, central regulation causes adiponectin knockout mice to have a phenotype with increased bone mass, associated with increased apoptosis of osteoblasts[150]. Adiponectin receptor expression is known to decrease in diabetes, and this decrease in type 2 diabetic Sprague-Dawley rats was shown to be attenuated through DPP4 inhibition, positively influencing their cognitive functions[151]. Possibly, DPP4 activity might similarly affect the adiponectin binding signal in the bone.

4.3. Ghrelin

Ghrelin is an appetite-stimulating hormone produced by the stomach, which signals hunger to the brain by regulating the hypothalamic expression of NPY, and is increased in a fasting state and decreased after food intake[152]. Although ghrelin is not a DPP4 substrate, DPP4 inhibition with sitagliptin was found to result in a suppression of active ghrelin[153]. In experimental studies, ghrelin stimulates osteoblast proliferation and differentiation going along with an increased concentration of bone formation markers in serum and BMD in femora of rats following infusion[154]. However, it was also found to increase bone resorption in rat osteoclasts and to increase bone turnover in a fasted state[155]. Ghrelin directly inhibits osteoclast progenitor differentiation but simultaneously stimulates osteoclastogenesis via a more potent central pathway independent of appetite regulation[156, 157]. A ghrelin knockout study indicated that ghrelin signaling is not crucial for normal bone development[158]. Leptin and ghrelin were found to interact strongly with each other. The ~~central~~ increase of osteoclastogenesis induced by ghrelin is suppressed by leptin, which balances these counteracting pathways and results in an unchanged bone structure. The bone protective effects associated to the interactions of leptin and ghrelin were found to be age-dependent, as the direct osteoclastogenic ghrelin pathway is lost with aging[156]. Furthermore, the contribution of ghrelin to BMD was found to be strongly influenced by hormonal status[159] as well as obesity[160]. In conclusion, the activity of ghrelin was found to have numerous effects on in the bone. Furthermore, the suppression of ghrelin induced by DPP4 inhibition could possibly affect bone turnover.

4.4. Insulin, amylin & preptin

Excretion of insulin by β -pancreatic cells is stimulated by nutrient ingestion through the action of gastro-intestinal hormones in a biphasic secretion process, with a first quick release of already synthesized insulin and a second longer-lasting phase of freshly produced insulin. Insulin, amylin and preptin are co-secreted by β -pancreatic cells and are all known to influence bone mass. Insulin is mitogenic to osteoblasts *in vitro* and increases OCN expression and bone formation *in vivo*, which could be mediated by insulin receptors on osteoblasts as well as IGF-1 receptors[161]. BMD was found to be directly correlated with circulating insulin levels and inversely correlated with insulin sensitivity[162, 163]. Hyperinsulinemia is the reason why high BMDs can be found in type 2 diabetes, and hypoinsulemia is mainly held accountable for a low BMD in type 1 diabetes[164]. Amylin is a peptide which directly stimulates the proliferation of osteoblasts *in vivo* and acts as calcitonin to inhibit osteoclast development and activity and thus bone resorption[165]. It was shown to positively affect bone mass in rodents[166]. Adrenomedullin is a strongly [amylin](#)-related peptide, also secreted by the β -pancreatic cells, which also exerts an anabolic effect on the bone[167]. The secretion of both amylin and adrenomedullin is dependent on the presence of the IGF-1 receptor[168]. Amylin has an effect on the growth plate and the linear growth of long bones similar to leptin[169]. Preptin is anabolic to osteoblasts in cell culture but does not influence osteoclastic activity. *In vivo*, it was shown to enhance bone formation in mice[170]. The effects of insulin, amylin and preptin on the bone could possibly be enhanced through the increase of the half-life of GIP and GLP-1 following the inhibition of DPP4 activity.

5. Summary of potential interactions of DPP4 in the bone

An overview of some of the interactions discussed above is displayed in table 1 and figure 24.

The interactions depicted are not established consequences of DPP4 inhibition, but potential interactions based on available literature.

Figure 24. DPP4, its substrates and their receptors on the surface of active bone cells. Normal arrows indicate binding, dotted arrows indicate a transfer, and '+' and '-' symbols indicate a potential stimulation and inhibition as determined by studies with individual substrates, respectively. LP: leptin, LPR: leptin receptor, APN: adiponectin, APNR: adiponectin receptor.

Table 1. DPP4 substrates and hormone peptides interacting with DPP4, that have receptors on surface of osteoblasts, osteoclasts and osteocytes, and the result of their binding activity as determined by independent~~separate~~ *in vitro/in vivo* experimental and clinical studies. DPP4i = *Potential* effect on cellular activity achieved by inhibition of DPP4 activity (? = neutral or unknown effect, - = negative effect, + = positive effect), BFR: bone formation rate, LPR: leptin receptor, APNR: adiponectin receptor, GHSR: ghrelin receptor, IR: insulin receptor. * not a DPP4 substrate. Receptors between brackets indicate an indirect effect (through neighboring cell or central regulation).

Substrate/ peptide	Receptor	Effect of binding	DPP4i
Osteoblasts			
GIP	GIPR	Increased ALP & Coll1 mRNA expression Improved mineralization, collagen maturity/fibril diameter Improvement of bone mineralization	+
GLP-1	GLP-1R (2)	Increase of OCN, OPG and RANKL expression Decrease of Runx2 expression Promotion of proliferation Inhibition of apoptosis Improved collagen maturity	+
SDF-1 α	(CXCR4)	Increase of osteoblast proliferation Increase of Coll1 expression	+
NPY/PYY	Y1	Decrease of BFR (and BMD) Decreased response to mechanical load induced BFR Reduced osteoblast differentiation Reduced bone formation	-
IGF-1	(Y2) IGF-1R	Increase of ALP, OPG and OCN Decrease of RANKL expression Stimulation of osteoblast proliferation/chemotaxis	+
VIP/PACAP	VPAC1/VPAC2	Increased osteoblast activity and bone noduli formation	+
SP	NK-1R	Stimulation of bone formation	+
LP*	LPR	Increase of OPG expression Decrease of RANKL expression	?
APN*	APNR	Increased osteoblast activity	+
GHS*	GHSR	Increased bone formation	-
Insulin*	IR	Increase of OCN, RANKL Increased bone formation	+
Osteoclasts			
GIP	GIPR	Inhibition of bone resorption	-
GLP-1	GLP-1R	Postprandial inhibition of bone resorption	-
GLP-2	GLP-2R	Inhibition of postprandial and nocturnal bone resorption	-
SDF-1 α	CXCR4	(Promotion of osteoclast differentiation) Inhibition of osteoclast apoptosis	+
NPY/PYY	(Y1)	Inhibition of (stress-induced) bone resorption	+
VIP/PACAP	VPAC1/PAC1	Transient inhibition and delayed stimulation of osteoclastic activity	?
SP	NK-1R	Increase of osteoclast activity and osteoclastogenesis	+
APN*	APNR	Inhibition of bone resorption	-
GHS*	GHSR	Increase of bone resorption and modulation of osteoclastogenesis	-
Osteocytes			

GIP	GIPR	Increase of collagen synthesis	+
GLP-1	GLP-1R	Inhibition of sclerostin synthesis	+
IGF-1	IGF-1R	Osteogenic response to mechanical loading	?

6. Effects of DPP4 inhibition on bone metabolism

In vivo and *in vitro* experimental studies regarding the effect of DPP4 inhibitors on bone metabolism have been inconclusive. In an experimental *in vivo* study published in 2010, the effect of sitagliptin on the bone was assessed in male high fat diet-fed type 2 diabetic mice[171]. Sitagliptin treatment effectuated an increase of vertebral BMD, trabecular architecture and bone mineral content compared to diabetic controls, however the observed effect in this study was associated with a significant reduction of glycemia. Furthermore, treated diabetic animals displayed a shift towards increased mineralization, which also resulted in a decrease of the Young's modulus. The genetic inactivation of DPP4, however, did not result in a specific bone phenotype[171, 172]. A more recent experimental study concluded that the experimental DPP4 inhibitor MK-0626 did not affect bone quality in type 2 diabetic muscle lysine to arginine (MKR) mice[173]. However, the effect of the mutation in the IGF-1 receptor in this model could be an influencing factor, as it was shown to reduce proliferation of bone cells as well as osteoblast activity[106], and also indirectly influence other incretins[174]. In a study conducted in streptozotocin-induced diabetic rats, DPP4 inhibition with sitagliptin was found to result in an attenuation of trabecular and cortical bone loss and an increase of mechanical bone strength, associated with an inhibition of bone resorption and independent of blood glucose levels[175]. In this study, the increase in MMP9 activity in diabetic animals compared to healthy controls was found attenuated in sitagliptin-treated diabetic animals[176]. *In vitro*, the blockade of DPP4 with a monoclonal antibody resulted in an inhibition of the p38 MAPK pathway (crucial for

osteoclastic differentiation) in human osteoclasts causing impaired osteoclast development, which is in agreement with the previously mentioned study[17]. In contrast, in another study, saxagliptin was found to inhibit insulin and IGF-1 and to decrease the production of Coll1 and expression of Runx2 and OCN in osteoblast-like cells[177]. The *in vivo* experimental part of that study revealed that saxagliptin treatment induces an increase in the serum levels of bone resorption markers, and a significant decrease of the osteocytic and osteoblastic density in trabecular bone and of the average height of the proximal cartilage growth plate in the femora of healthy rats[177]. Experimental studies assessing alveolar bone of rats with periodontitis did not reveal any influence of sitagliptin on alveolar bone loss, but reported a decreased MMP9 gene expression[178]. Clinical studies evaluating the effect of DPP4 inhibition on the bone have also been inconclusive. A *post hoc* pooled analysis associated the use of saxagliptin with a higher incidence rate per 100 person-years for bone fractures in diabetic patients[179]. In contrast, an earlier clinical meta-analysis of trials with a duration of more than 24 weeks involving over 20,000 type 2 diabetic patients, published in 2011, suggested a protective effect of DPP4 inhibitors on bone fractures. It should be noted that despite the high number of participants, the numbers of fractures was surprisingly low, as only 63 fractures were reported and the study only included bone fractures reported as serious adverse events[30]. In a case-control study conducted by Driessen *et al.*, the use of incretins was not associated with a change of fracture outcome after 1.3 years of inhibitor use[180]. A recent case-control study conducted by the same group in which following-up patients were followed-up over a period of four years did not reveal any associations between the use of DPP4 inhibitors and fracture risk[181]. Bone resorption markers as well as calcium homeostasis were found unchanged in a study in type 2 diabetic patients revealing no influence of one year of vildagliptin treatment on bone metabolic markers in the

serum[182]. In postmenopausal type 2 diabetic women, however, a 12 week treatment with sitagliptin was associated with a decrease of bone resorption markers[183]. To the best of our knowledge Unfortunately, long-term studies evaluating the about the effects of DPP4 inhibitor intake on bone metabolism have not been executed. Hence, at this moment, do not yet exist, so no long-term conclusions on the long-term effects of DPP4 inhibition on fracture outcome can be drawn-at this moment[184].

7. Summarizing and concluding remarks

This review gives a concise summary of the effects of the different DPP4 substrates and related proteins on the bone. Taking into account the effects of a list of (important) DPP4 substrates (GIP, GLP-1, GLP-2, IGF-2, VIP and PACAP), a protective effect of DPP4 inhibition on bone may reasonably be assumed. On the other hand, for a nearly as long list of substrates (SDF1 α , NPY, PYY and substance P) the potential effects on the bone are less straightforward. The insulinotropic gastrointestinal peptides GIP[39] and GLP-1[29], as well as GLP-2[71], released in the splanchnic circulation after food intake, are able to reach the microcirculation of the bone where they play a well-documented role in the promotion of bone formation and/or the inhibition of bone resorption. In the bone, neuropeptides secreted from local skeletal nerve fibers like VIP[122, 125], PACAP[124], NPY[185], PYY[101], SP[122] and IGF-1[108], influence the activity of osteoblasts and osteoclasts directly. Besides having a direct effect on the activity of osteoclasts, VIP also exerts an immunoregulatory role and NPY and PYY are also known to affect bone metabolism through feeding and energy regulation[84, 186]. NPY, involved in many metabolic pathways, is produced within the bone by osteoblasts as well as osteoclasts. Although

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9 in theory, DPP4 inhibition would increase signaling by NPY and PYY through the Y1 receptor
10 resulting in a direct inhibitory effect on osteoblasts, they also exert a more complex indirect
11 effect through the hypothalamus[92]. The growth factor IGF-1, which stimulates osteoblastic
12 proliferation and inhibits bone resorption, as well as the chemokine SDF-1 α , the most efficiently
13 processed DPP4 substrate which plays an important role in the differentiation of active bone
14 cells, both are also produced locally by osteoblasts and osteocytes. All of these DPP4 substrates
15 thus contribute to the effects of DPP4 inhibition on the bone. Any attempt to elucidate
16 mechanisms underlying the effect of DPP4 inhibition on the bone seems primarily related to the
17 reduction of bone resorption. Unfortunately, this does not allow to narrow down the contribution
18 of DPP4 substrates to individual peptides, but rather suggests a sophisticated and well-
19 orchestrated cumulative effect. This, together with the fact that the DPP4-related activity of
20 adipokines also has a divergent effect on bone metabolism makes it difficult, if not impossible, to
21 predict the potential overall effect of DPP4 inhibition on the bone. This is also reflected by the
22 results of a significant number of experimental as well as clinical studies pointing to a beneficial
23 shift of DPP4 inhibition on bone homeostasis (which however needs to be confirmed on the long
24 term), whilst other studies reported either opposite results or no effect of DPP4 inhibition on the
25 bone[30, 171, 173, 175, 177, 180, 181, 184]. Many pathways could be involved, but their
26 individual contribution is yet to be established. For each of the previously discussed actors
27 associated with DPP4 (inhibition) and the bone, the origin of expression, the range of action
28 considering their secretion and journey, and the receptor or pathway through which their function
29 is effectuated have to be taken into account. With regard to the substrates, the affinity of DPP4
30 but also the organs in which they are synthesized as well as their exposure to DPP4 in their
31 journey towards the bone and their affinity to their receptors have to be taken into account in the
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9 evaluation of their possible effect. An important problem in the assessment of the potential
10 contribution(s) of particular DPP4 substrates is the precise distinction between active and non-
11 active (truncated or modulated) forms of DPP4 substrates with detection assays. As binding
12 activity of substrates can be dependent on two N-terminal peptides, analytical development of
13 these assays has proven to be a tough challenge. The expression of substrate receptors can be
14 variable according to region or developmental phase. Furthermore, it is important to mention that
15 effects of DPP4 inhibition on the bone can importantly be modified by underlying
16 pathophysiological processes in energy metabolism and the bone i.e. diabetes[56], osteoporosis
17 (menopause)[26, 86, 187], obesity[7]. Therefore, as an overall conclusion, it could be stated that
18 the potential effect of DPP4 inhibition on the bone should be evaluated during long-term clinical
19 studies in relevant subject groups (healthy volunteers as well as obese, diabetic and osteoporotic
20 patients) in order to be able to better comprehend effects of DPP4 inhibition on the bone.
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