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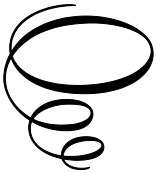

Integrative Review of Ghrelin Family Peptides, Musculoskeletal Health and Osteoporotic Fractures

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In blessed memory of Abraham (Buma) and Lea (Lisa) Fisher

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PREFACE

In this review, we aim to summarise the existing data on the direct and indirect relationships between ghrelin family peptides (GFPs) on the musculoskeletal system and their contribution to osteoporosis (OP), falls, and, consequently, to osteoporotic fractures (OFs). We briefly describe the cells producing acylated and unacylated ghrelin, obestatin (all three peptides are products of the same preproghrelin gene), and nesfatin-1 (which is produced by the same cell), their local and systemic pluripotent effects under physiological conditions, and focus on emerging data regarding the potential contribution of these peptides to OP/OFs. We provide a conceptual basis to consider dysregulation in the production, release, and action of these peptides as important (patho)physiological components in the complex network involved in musculoskeletal health and discuss the possible role of GFPs, mimetics, and their receptors as therapeutic targets for the prevention and treatment of OP/OFs and numerous chronic diseases recognised as high risk for falls and fractures. We formulate several challenging questions and, despite the limited evidence, present advice to implement the evaluation and use of GFPs in clinical practice based on emerging data on their potential diagnostic and therapeutic benefits in different disorders. An extensive bibliography is provided.

Keywords: bone, muscle, ghrelin, obestatin, nefstatin-1, osteoporosis, fracture, chronic diseases

INTRODUCTION

Osteoporotic fractures (OFs), the dramatic outcome of bone fragility and falls, are a major public health challenge worldwide. Despite intensive research and currently used multiple diagnostic, preventive, and therapeutic interventions, the incidence of OFs continues to rise as the population ages, and predictions for the coming decades are alarming [1-5]. As both osteoporosis (OP) and falls resulting in OFs have a high level of genetic predisposition (above 50%), identification and addressing non-heritable and modifiable determinants for OFs is of paramount importance.

The maintenance of bone and muscle homeostasis* is tightly controlled by many organ systems affecting, in different ways, numerous metabolic pathways. Among these, the essential role of the endocrine organs (hypophysis, gonads, parathyroid, adrenal, pancreatic, and thyroid glands) is well established, while the contribution of gut hormones remains only partially characterised.

Although the gut is the largest endocrine organ in our body [10], and the list of identified gut hormones is growing (more than 20), comparatively little attention has been paid to the research on the stomach/gut hormones–musculoskeletal axis. Numerous data indicates that gut hormones not only orchestrate the body's response to food ingestion, regulating key physiological processes involved in gastrointestinal functions, but that they also play important roles in the coordinated control of metabolism outside the gut. Most previous reviews concentrated on and critically assessed the relationship between gut-derived hormones, such as gastric inhibitory polypeptide (GIP) and glucagon-like peptide (GLP-1 and GLP-2), and bone [11-17]. In this review, we focus on four peptides – acyl ghrelin (AG), des-acyl ghrelin (DAG), obestatin (Ob), and nesfatin-1

* In recent years, the term 'homeostasis' has become controversial; to avoid ambiguity, misunderstanding, and different epistemologies, in this article we use this term, in contrast to the canonical view, in a more contemporary and broader sense to mean all types of integrated physiological regulations (considering adaptive dynamic equilibrium [proper homeostatic balance], homeoresis, allostasis, and anticipatory control) generally involved in the ongoing maintenance, stabilisation, and coordination of vital physiological parameters to the levels optimal for survival and adaptation to the changing environment [6-9].

(Nesf-1), further referred as the 'ghrelin family peptides' (GFPs).† These peptides are produced mainly in the stomach [18-20] by the same enteroendocrine cell (the first three peptides are encoded by the same preproghrelin gene) and also widely expressed in the body. Accumulating evidence indicates the importance of these four peptides in the cellular signalling networks that directly and indirectly control, among other functions, the musculoskeletal metabolism under physiological conditions and, when dysregulated, may negatively affect bone and muscle status predisposing one to OP, falls, and OFs.

Published reviews have either examined the role of individual GFPs on bone or muscle health or their relationship with specific diseases and potential therapeutic use. Despite the growing interest in GFPs, their physiological roles and the potential impact of dysregulation(s) on OP/OF have not been fully elucidated; integrative studies investigating their pathophysiological role in OP/OF are lacking. Consequently, assessment of GFPs' status and corrections of their alterations as a part of management strategies for the prevention and treatment of OP/OFs are not incorporated into current clinical practice. Our review is focused on an integrative approach involving the physiopathological effects and interactions of the four GFPs on the musculoskeletal and other organ systems relevant to OP/OFs.

The review is organised as follows. First, we briefly present the general characteristics of GFPs, the spectrum of their functions, and their regulation under physiological conditions. Second, we describe the relationships between these hormones and musculoskeletal status in states of health and disease and discuss the mechanistic basis of these relationships. Third, we briefly characterise the genetic, environmental, lifestyle, epigenetic, pathological, and drug-related factors that may affect GFP production, release, and action. Fourth, we present the potential effects of GFP dysregulation on diseases and disorders known to be associated with OP/OFs. Finally, we discuss practical applications of the new data to OP/OF management, highlighting the diagnostic, preventive, and therapeutic potential of GFPs and the available analogues, receptor agonists, and antagonists in different settings. We illustrate a practical approach to incorporating the new data in clinical practice and end with a summary of key findings.

† Ghrelin family peptides (GFPs) is used here as an umbrella term to include AG, DAG, Ob, and Nesf-1; the term ghrelin gene peptides (GGPs) indicates AG, DAG, and Ob (which are all produced by the same gene).

When integrating different aspects of this broad topic (a search of Medline using only the term ‘ghrelin’ revealed more than 11, 000 papers) for the first time, in the interest of keeping the review of modest length and readable for a wider audience, we present a significant number of articles and relevant information mainly in the tables and summarise the data without detailed comments; it should be noted that this not a formal systematic review.

CHAPTER 1

OVERVIEW OF THE GHRELIN FAMILY PEPTIDES (GFPS)

1.1. Cells producing GFPs: Anatomical distribution and hormonal products

Ghrelin was originally discovered and purified from the rat stomach in 1999 [18] as an intrinsic ligand for the growth hormone (GH) secretagogue receptor 1a (GHS-R1a) and is a 366-amino acid protein (M_r = 31, 329); this is reflected in its name: ***g***rowth ***h***ormone ***rel***easing ***in***ducing **=** ghrelin [21]; the word root ['*ghre*-'] in proto-Indo-European languages means 'grow'. The gastric ghrelin-producing cells (P/D_1 in humans, X/A-like cells in rodents and dogs) are located mainly in the basal portion of the oxyntic glands and in lower numbers in the antral mucosa [22-28] in proximity to enterochromaffin-like cells (ECL) and represent the second most prevalent endocrine cell population (20–30%) after the ECL (30–50%) [28-36]. Ghrelin expression gradually decreases from the duodenum (the second most crucial ghrelin-producing site) [37] to the colon [29, 36, 38-46].

It is worth noting that the old paradigm and classification of the enteroendocrine cells secreting particular hormones (one cell – one hormone) [47-49] has recently been reconsidered, and a novel concept of enteroendocrine cell hormonal plasticity has been introduced [50]. It has been revealed that individual enteroendocrine cells co-express/co-secrete variable mixtures of peptides with approximately a 6 to 20% overlap in the hormonal spectrum [26, 51-54]. The hormonal repertoire of these cells is determined and modulated by their location along the gastrointestinal tract, diet, metabolic state, and by multiple signalling molecules, including bone morphogenetic proteins (BMPs) [55, 56]. The ghrelin-producing X/A cells in the small intestine also express cholecystokinin (CCK), secretin, motilin, somatostatin, pancreastatin, and proglucagon [28, 57, 58], whereas I cells, which mainly secrete CCK, store ghrelin, glucose-dependant insulinotropic polypeptide/gastric inhibitory polypeptide (GIP), glucagon-like peptide 1 (GLP-1), peptide Y (PYY), secretin, proglucagon, and

neurotensin [51, 52, 59]. GLP-1, PYY, and oxyntomodulin are predominantly secreted from intestinal L-cells; GIP is secreted from endocrine K cells in the intestinal epithelium. Similarly, the hypothalamic cells co-express ghrelin, Nesf-1, and other peptides involved in the regulation of food intake (melanin-concentrating hormone, alpha-melanocyte-stimulating hormone [α-MSH, anorexigenic], proopiomelanocortin, neuropeptide Y [NPY, orexigenic], and cocaine- and amphetamine-regulated transcript), pituitary hormone secretion (growth hormone-releasing hormone [GHRH], corticotropin-releasing factor [CRF], thyrotropin-releasing hormone [TRH], vasopressin, oxytocin, and somatostatin), and in stress response (CRF, serotonin, dopamine, neurotensin, neuronostatin, and urocortin) [20, 60-73].

The gastric GFP-producing cells, in contrast to most enteroendocrine cells (e.g., CCK, GLP-1, PYY), are closed-type (they do not directly contact with the gastric lumen) and differ from the intestinal ghrelin cells; the rodent stomach also has open-type cells containing only DAG [22]. In the small and large bowel, there are both closed- and open-type GFP-secreting cells [18, 29]. Gastric ghrelin cells express mRNA ghrelin at a level about threefold higher than any other peptide hormone mRNA and do not express motilin [51, 74].

Ghrelin is secreted predominantly in the stomach (60–70%) and released into circulation [18, 42, 75-80]. Gastrectomy decreases circulating ghrelin levels by about 80 per cent in both humans [77, 81] and rats [82]. Bariatric surgery (Roux-en-Y gastric bypass, sleeve gastrectomy) also causes a large reduction in ghrelin cell counts, secretion, and circulating levels of ghrelin [24, 83-85].

To a lesser extent than in the stomach and intestine, ghrelin is also widely expressed and produced in various organs: different brain areas (particularly in the arcuate nucleus of the hypothalamus), endocrine glands (anterior pituitary, pancreatic islets [epsilon cells], adrenals, thyroid, ovaries, testes, placenta), bone, muscle, cardiomyocytes, adipocytes, lymphoid/immune, liver, heart, kidney, lung, and sebaceous gland cells [29, 36, 42, 59, 86-116].

Acyl ghrelin, des-acyl ghrelin, and obestatin. The human ghrelin gene is located on chromosome 3p25-26 [117] and contains four preproghrelin-coding exons. From the 117-amino-acid preprohormone precursor (in both humans and rats), three peptide hormones are derived by post-translational alternative mRNA splicing and/or proteolytic processing [19, 118-123]: (1)

acyl ghrelin (AG), a 28-amino-acid peptide with an *n*-octanoyl group on the third serine residue (in all vertebrates except the bullfrog, which has threonin); (2) des-acyl ghrelin (DAG), a 27-amino-acid peptide derived from the C-terminal part of the preproghrelin [38, 124, 125]; and (3) obestatin (Ob, named due to its inhibitory effect on food intake [120, 126]), a 23-amino-acid C-terminally amidated peptide that forms an α-helix.

Preproghrelin is acylated with an octanoyl chain that is unique among known proteins by ghrelin O-acyltransferase (GOAT), an enzyme found predominantly in the stomach [112, 127-133]. The acylation is essential for activation of the ghrelin receptor GHS-R1a and its main physiological effects [127, 129, 130, 132, 134-139]. This transmembrane G protein-coupled receptor is most highly expressed in the stomach but also widely expressed in the hypothalamus and other regions of the brain, the anterior pituitary, thyroid, and adrenal glands, the gonads, bone, the myocardium, the lungs, the pancreatic islets, the gut, the kidneys, immune cells, and adipose tissue [89, 117, 129, 140-149]. The biological importance of GGPs is further indicated by evolutionary studies which showed that the AG–GHS-R1a signalling pathway has been conserved for about 400 million years [134, 150-152]; among most vertebrate species, the N-terminal 10 amino acids in AG are identical, as well as the functional key residues in all GHS-R isoforms [153-155].

The total ghrelin concentration in human plasma is around 100–150 fmol/ml [140]. AG is cleaved by serum esterases (mainly by butyrylcholinesterase in humans, carboxylesterases in rodents) [156-162] to DAG. Ghrelin desoctanoylation in serum is significantly slower in humans (236 ± 18 min) than in rodents (27 ± 2 min) [156]. The kidneys and liver play a role in the clearance of ghrelin [163, 164]. AG and DAG have distinct rates of clearance: AG disappears more rapidly from plasma than DAG [165]. In pharmacokinetics studies, the half-life [$t_{1/2}$] ranged for AG between 9 and 13 min [165, 166] and 21 ± 3.0 min [167] and for total ghrelin between 27 and 36 ± 2.4 min [165-168]. The shorter $t_{1/2}$ of AG reflects its quick deacylation in the circulation and conversion to DAG, while the $t_{1/2}$ of DAG depends mainly on the clearance of the peptide from the circulation [166]. Circulating ghrelin is protected from degradation, at least partially, by binding to immunoglobulins (Ig) [169, 170].

Most studies based on ghrelin measurements by immunoassays (ELISAs) demonstrated that ghrelin in the circulation exists mainly in the form of DAG, but some researchers have interpreted these results as an error/artefact in collection and analytical techniques and postulated that

AG is total ghrelin and DAG should not be detectible in healthy human plasma under optimal sample handling and assaying conditions [171]; recently, the bioanalytical procedures for the quantification of AG and DAG have improved [172-174].

Notably, most of the central and peripheral ghrelin's effects have initially been attributed to the actions of AG, whereas DAG, the most predominant isoform of ghrelin in serum (80–90%) [77, 146, 175], which does not activate the GHS-R1a [161, 176-178], was thought to be purely an inert degradation product of AG [38]. However, further research clearly demonstrated that DAG exerts multiple important biological activities participating in many physiological and pathological processes (through an unidentified receptor, distinct from GHS-R1a) [161, 178-182]. DAG is considered a separate hormone acting independently or together with AG [175, 183, 184].

Although a substantial number of investigators concluded that two forms of circulating ghrelin (AG and DAG) may have distinct roles in a variety of physiological functions (see Table 1, below), a large proportion of studies measured and referred to plasma ghrelin without specifying the acylation status. Because many articles failed to distinguish AG from DAG, throughout this review we refer to 'ghrelin' if the peptide's isoform has not been specified in the cited study and use the terms 'AG' and 'DAG' when it was reported.

As mentioned above, Ob is produced in the same endocrine cell type and generated from the same prohormone as ghrelin [33, 119, 126, 185-191], stored in the same cellular secretory vesicles [187], and expressed throughout the gastrointestinal mucosa (from cardia to ileum) as well as by several other tissues, including the pancreatic islets [185-187, 189, 192], liver [186], spleen, mammary gland [187, 193], thyroid [194], lung [189], kidney [195], testis [196], adipocytes [197], breast milk, and plasma [187, 198], indicating in addition to endocrine its local autocrine/paracrine actions. The half-life of exogenous Ob (10 micrograms iv) in rodents is about 22 min [199]. Despite its short biological half-life and rapid degradation [200, 201], Ob exerts multiple physiological actions. The main organ source of the ghrelin-gene products (AG, DAG, Ob) may be different: after gastrectomy, plasma ghrelin levels decrease dramatically, while decreases in Ob levels do not reach statistical significance [202], indicating that more Ob originates possibly outside the stomach (in the small intestine and/or the pancreas).

Nesfatin-1. Further complexity was added to GFP physiology with the finding that gastric ghrelin-producing cells in both humans and rats [203]

are the main source of peripheral nesfatin-1 (Nesf-1), the N-terminal 82-amino acid biological active polypeptide fragment derived from the 396-amino acid precursor protein nucleobindin 2 (NUCB2) [20, 68, 204-211]. While AG, DAG, and Ob are encoded by the same gene (*GHR)*, Nesf-1 is produced by the *NUCB2* gene [203] and stored within different vesicles [212]. The 29-amino-acid mid-fragment of Nesf-1 (nesfatin-1: 30–59) has been identified as the active core of Nesf-1 [206, 211]. First isolated in the brain, Nesf-1, similarly to three other GFPs, was found to be broadly expressed in various tissues and organs, including the stomach, small intestine, bones, skeletal muscle, brain (hypothalamus, amygdala, brainstem), spinal cord, endocrine system (pituitary gland, adrenals, pancreatic beta cells, ovaries, testes), adipose tissue, heart, lungs, liver, kidneys, and saliva [20, 205, 206, 213-226]. The stomach was identified as the main source of peripheral Nesf-1 with mRNA levels higher than in other peripheral organs or the brain [203]. In rats, Nesf-1 expression levels in the gastric oxyntic mucosa are 10-fold higher compared to the brain [219]. The half-life of NUCB2 mRNA is approximately six hours [227]; the half-life of Nesf-1 is 9–10 [228, 229] ∅3.5 minutes [230]. Similarly to ghrelins (GGPs), the expression and biological actions of Nesf-1 are also highly conserved across species [231].

The mouse gastric ghrelin cells also synthesise and secrete into the blood other proteins (retinol-binding protein, transthyretin) [232], which may interact with the biological activities of GFPs.

In humans and rodents, nesfatin-1, an 82-amino-acid peptide weighing 9.8 kDa, is encoded in the N-terminal region of the nucleobindin-2 gene (*NUCB2*). The product of the *NUCB2* gene is a 420-amino-acid (aa) peptide composed of a 396-aa long precursor peptide and a 24-aa long signal peptide. Proteolytic enzymes (convertase PC1/3 and PC 2) cleave the NUCB2 peptide into three parts: nesfatin-1 (1–82 aa), nesfatin-2 (85–163 aa), and nesfatin-3 (166–396 aa). The midsegment of nesfatin-1 (30aa) is its bioactive core responsible for multiple diverse physiological functions. The biological role of C-terminal fragments of NUCB2 (nesfatin-2 and nesfatin-3) remains unknown, and the receptor for nesfatin-1 is still unidentified. NUCB2 and nesfatin-1 are co-localised, and their expression is often analysed together.

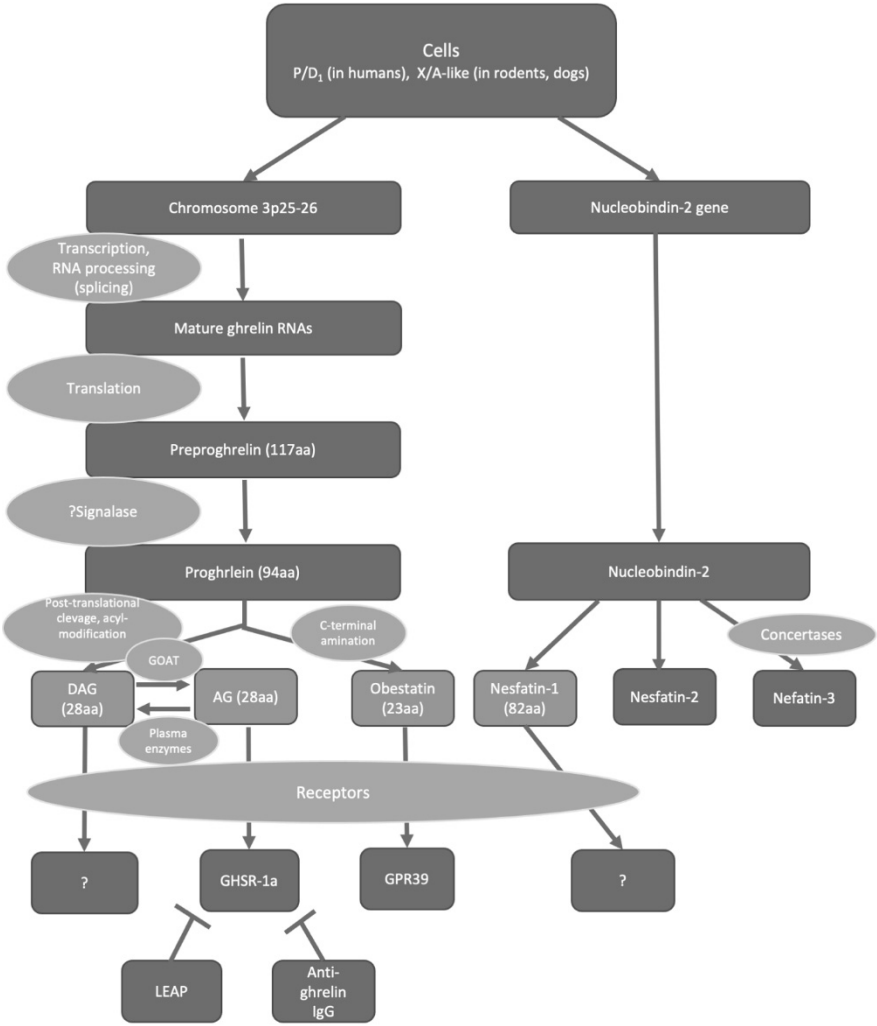

Figure 1. Simplified diagram summarising synthesis of three ghrelin peptides (encoded by the ghrelin gene) and nesfatin-1 (encoded by nucleobindin-2 gene) and their receptors.

Abbreviations: aa, amino acids; AG, acylated ghrelin; DAG, des-acylated ghrelin; GH, growth hormone; GHSR-1a, growth hormone secretagogue receptor 1a; GOAT, ghrelin O-acyltransferase; GPR39, orphan G protein-coupled receptor; IgG, immunoglobulin G; LEAP, liver-expressed antimicrobial peptide 2.

Notes. These four peptides are synthesised by P/D_1 (in humans) or X/A-like (in rodents, dogs) cells of the stomach's oxyntic glands (mainly) and many other tissues including gut, brainstem (hypothalamus, ventrolateral medulla, pituitary gland, spinal cord, etc.), pancreas, kidney, heart, lung, adrenals, ovaries, spleen, placenta, and subcutaneous fat tissue. Acylated ghrelin (AG), des-acylated ghrelin (DAG), and obestatin (Ob) are derived from the same ghrelin gene. In the endoplasmic reticulum, post-translational processing of the 117-amino-acid preproghrelin peptide forms proghrelin (94aa), which is subsequently cleaved by convertase 1/3 resulting in the formation of non-acylated ghrelin (28aa) on the NH_2-terminal end. The enzyme ghrelin O-acyltransferase (GOAT) acylates serine3 residue in the 28-amino-acid polypeptide chain producing the mature form of AG; the n-octanoylation of serine is essential for AG binding to the GHSR-1a and, consequently, for the majority of its biological functions. Obestatin (Ob, a 23aa) is cleaved from the 66-amino-acid COOH-terminal end of proghrelin (or a product of alternative gene splicing). The ghrelin gene locus is complex, and currently the ability to quantify each form of ghrelin by standard immunoassay is limited. After secretion, AG activates GHSR-1a; the receptor for DAG (> 90% of circulating ghrelin) is still unidentified. The proposed receptor for Ob is GPR39. In circulation, AG rapidly becomes deacylated by plasma enzymes (i.e. carboxylesterases, butyrylcholinesterse, platelet-activating factor acetylhydrolase, carboxypeptidase, etc.) and forms DAG. LEAP (a 40-residue cationic peptide produced predominantly in the liver and small intestine) binds to GHSR-1a and acts as its antagonist.

The NH_2-terminal ghrelin cores with acyl-modification sites and the nesfatin-1 (especially its mid-segment) are well conserved across all vertebrate classes.

1.2. Regulatory factors, receptors, and major physiological functions

Regulatory factors. The GFP-producing cells in the gut, like most enteroendocrine cells, are delicately controlled by complex mechanisms involving interactions between neural, endocrine, autocrine, and paracrine pathways with multidirectional signalling networks [53, 146, 233-235]. Host genetic factors, different nutrients, chemosensory signalling, and vascular and luminal (including gut microbiota and inflammatory-derived) stimuli directly and indirectly acting on GFP-producing cells affect synthesis and release of the hormones, their plasma levels, and subsequent feedback.

Biosynthesis and circulating concentrations of GFPs exhibit an ultradian pulsatility, and plasma levels vary with the nutritional status. Fasting results in significantly elevated secretion of both AG and DAG [30, 42, 91, 236-246], reduced Ob [199, 247-250] and Nesf-1 levels [20, 206, 251-254], whereas after meals circulating levels of these peptides (along with CCK,

gastrin, somatostatin, GLP-1, glucose-dependent insulinotropic peptide [GIP], peptide tyrosine tyrosine [PYY(3–36)], oxyntomodulin [OXM], gut-derived serotonin [5-hydroxytryptamine, 5-HT], insulin, and pancreatic glucagon) changes in opposite directions [53, 61, 147, 210, 243, 255-263]. In other words, after nutrient ingestion, ghrelin levels, in contrast to all other appetite-regulating hormones, rapidly decline and increase again gradually, reaching a peak immediately before next meal. GOAT expression and acylation also vary with nutritional status, caloric load, and macronutrient composition (e.g., fatty acids, glucose intake) [136, 234, 264-266].

The increase in ghrelin secretion following an overnight fast is mediated by sympathetic stimulation (norepinephrine acts on β1-adrenergic receptors, which are the most highly expressed within ghrelin cells [267]) [155, 267-272]. Parenteral and intracerebroventricular administration of ghrelin reduces plasma norepinephrine levels and inhibits sympathetic activity [273-275]. The vagus nerve and the cholinergic system are also important for increasing fasting ghrelin levels [234, 241, 276-278]. Meal intake suppresses ghrelin-producing cells by vagal activation [279, 280], high-fat diet [281, 282], rise in glucose [266, 271, 283-286], medium- and long-chain fatty acids, and amino acids [146, 282, 287-289]. These effects are also mediated by multiple inputs from the gut and systemic hormones including insulin [28, 148, 271, 290], glucagon [271, 291], somatostatin [292-297], CCK [256, 298-302], GLP-1, PYY [256, 303], GH [304], leptin [304-307], cortisol [308], oxytocin, oestrogens [167, 309], dopamine [146, 267, 283, 310-312], and serotonin [257, 313]. The release of AG is not affected by intragastric pH, while the release of DAG from the perfused stomach is greater at pH 2 than at pH 4 [22]. It should be noted that although the four GFPs – AG, DAG, Ob, and Nesf-1 – are co-expressed in the same cells, the regulating stimuli may be different.

Recently, it was discovered that liver-expressed antimicrobial peptide 2 (LEAP2) antagonises the ghrelin receptor GHSR-1a [314, 315]. LEAP2 is thought to be a more conserved ligand than ghrelin for fish GHSRs [316]. LEAP2 (in mammals mainly produced in the liver but also in the small intestine and co-expressed with ghrelin and pancreatic polypeptide in islets) fully inhibits GHSR1a activation by ghrelin and blocks the major effects of ghrelin in vivo, including food intake, GH release, maintenance of glucose levels during chronic caloric restriction, and body temperature reduction. Neutralising antibodies that block endogenous LEAP2 function enhance ghrelin action [131, 317-328]. The inhibitory effect of LEAP2 appeared to be specific for GHSR-1a as it did not inhibit DAG-induced reduction in body temperature or neuropeptide Y (NPY)-induced food

intake [321]. Circulating levels of LEAP2, in contrast to ghrelin, are suppressed by fasting and increase postprandially [131]. In patients with rheumatoid arthritis, serum LEAP2 levels were found to be significantly higher compared with controls and correlated with inflammatory cytokines including IL-6, IL-8, IL-1β, and CRP [329].

Receptors. GFPs display broad biological activities on both peripheral and central tissues involving distinct yet not fully identified receptors and modulating cofactors. To date, a receptor has been identified only for AG – a G-protein coupled growth hormone secretagogue receptor (GHSR); its encoding gene is located at chromosome 3q26.31. There are two receptor isoforms: GHSR-1a (functional, binds AG) and GHSR1b (biologically inactive, but possibly modulates GHSR-1a receptor activity) [134, 141, 152, 162, 330-335]. Notably, effects of ghrelin were observed after the GHSR gene had been inactivated, as well as in cells/tissues lacking GHSR-1a [333, 336-338]. The ghrelin receptor is highly constitutively active [339], may be regulated independently of ghrelin (e.g., heterodimerisation with other G-protein coupled receptors – dopamine receptors 1 and 2), and activates several different signalling pathways on ligand stimulation [340]. It has been hypothesised that direct effects of ghrelin on osteoblast proliferation, myoblast differentiation, insulin release, adipocyte lipid accumulation, cardioprotection, coronary artery constriction, vascular endothelial cell proliferation, and tumour cell proliferation may be mediated not only through GHSR-1a receptors [333, 336-338, 341-346] but also through ghrelin receptor-like receptors (GRLRs) or DAG receptors [333, 338].

As DAG does not bind GHS-R1a [38, 347] or binds the AG receptor with a lower affinity [347-351] and acts on cells lacking GHSR-1a [125, 183, 184, 342, 352-354], its widespread physiological effects indicate the existence of a still unknown specific receptor distinct from GHS-R1a [87, 333, 338, 355-361] or interaction with the AG receptor [362]. The biological actions of DAG might be also explained by the GOAT activity [360]. Because GOAT is expressed in most peripheral tissues, it is possible that locally produced DAG can be acylated [363].

The receptors that mediate the effects of Ob [122, 198, 364-369] and Nesf-1 [68, 370-372] are currently unknown; studies point towards a widely distributed in peripheral organs G protein-coupled receptor [72, 221, 373-375]. At the hypothalamic level, Ob inhibits ghrelin-induced expression of neuropeptide Y (NPY) and NPY receptors [210, 255]. Ghrelin receptor (GHS-R1a) is required for the effect of Nesf-1 on food intake and glucose

metabolism [376]; positive feedback between Nesf-1 and CCK has been shown in fed and unfed goldfish [377].

Major physiological functions. GFP-producing cells release their products in an endocrine, paracrine, and autocrine fashion; therefore, these hormones act on distant organs and locally. However, it is difficult to estimate to which extent the peptides' effects are driven by the endocrine or autocrine/paracrine action.

The AG, DAG [117, 121, 378-383], and Nesf-1 [228, 229] cross the blood–brain barrier bi-directionally and affect different brain zones [384-390]. The role of the blood–brain barrier regarding Ob is controversial [190]; this peptide was found to be biologically active in the brain in some [196, 199, 391, 392] but not all [124, 393, 394] studies.

GFPs play a pivotal role in the complex gut–brain network as integral regulators of food intake (homeostatic and hedonic) and energy balance [146, 155, 162, 235, 244, 256, 259, 260, 263, 395, 396]. Ghrelin is the only known peripherally produced and centrally acting orexigenic hormone, leading to an increase in food intake and body weight, whereas all other gut hormones (Ob, Nesf-1, CCK, GLP-1, pancreatic polypeptide [PP], oxyntomodulin) and adipokines (e.g., leptin, which originates mainly from the adipose tissue; only a minority of leptin is produced by the chief cells of the gastric fundic glands) are anorexigenic, leading to a decrease in food intake. The balanced GFP activity reflects the evolutionary selection processes for survival when caloric supply is reduced and the energy balance becomes negative [89, 146, 235, 256, 259, 260, 263, 397-405].

GFPs participate in controlling energy balance and body composition through the regulation of appetite, feeding behaviour, thermogenesis, glucose and lipid metabolism, gastrointestinal motility, secretion, digestion and absorption of many ingested nutrients (including calcium, phosphates, vitamins, and other nutrients involved in bone and muscle metabolism), and gut microbiota, conveying to the central nervous system (CNS) information concerning the nutritional and metabolic status and integrating the signals to the hypothalamic nuclei (directly affecting the release of orexigenic neuropeptide Y (NPY]) and agouti-related protein (AGRP) with concomitant inhibition of the anorectic pro-opiomelanocortin [POMC] and cocaine-and-amphetamine responsive transcript (CART) neurons [104, 155, 244, 262, 398, 400, 406-408]. The effects of GFPs on coordinated feeding response require intact vagal afferent nerves; ghrelin and possibly Ob stimulate vagal afferent (sensory) pathways from the gut

[155, 409-416]. GFPs regulate the action of each other and act via multiple pathways in concert with most gastrointestinal and systemic hormones, adipocytes (leptin, adiponectin), and neuronal signals, adapting the body's response to environmental, nutritional, stress, and inflammatory related challenges [26, 121, 210, 255, 417-429].

GFPs are multifunctional regulators of cell biology and are implicated in a plethora of physiological functions, apart from the regulation of food intake. GFPs affect most organ systems, including the musculoskeletal, digestive, nervous (modulating behaviour, sleep wake rhythm, memory, cognition, emotions, stress, anxiety), cardiovascular, renal, respiratory, endocrine, and reproductive systems (Table 1). It should be acknowledged that the GFPs–metabolism and GFPs–organ system axes represent bi- and multidirectional communication routes. GFPs affect multiple organs and, in turn, molecules released from different organs influence the function of cells producing GFPs (as well as other physiological mediators). For example, ghrelin is an important modulator of insulin and glucagon secretion, glucose, fat, and protein metabolism, and all these factors, in turn, are among the major determinants of ghrelin secretion [42, 271, 430-432].

Although all four GFPs are produced by the same cell (three of them, namely AG, DAG, and Ob, are products of the same gene and have a common precursor) and are thought to be secreted concomitantly, their biological activities differ from each other. Similarities and differences in metabolic actions and effects of different GFPs are shown in Table 1. It appears that DAG, Ob, and Nesf-1 antagonise some AG effects, especially on appetite, energy balance, and glucose metabolism, but act independently or together with AG on other functions, including cellular and metabolic pathways. For example, in some conditions (both in vitro and in vivo), DAG counteracts AG activities [175, 423, 433-435], whereas both AG and DAG promote the proliferation and differentiation of osteoblasts and skeletal myoblasts [87, 91] as well as affecting numerous other cell types (including pancreatic β cells, endothelial cells, preadipocytes, embryonic stem cells, cardiomyocytes, and adrenocortical tumour cells [436-442].

Understandably, in clinical analyses, both differential and synergistic influences of AG, DAG, Ob, and Nesf-1 on metabolic, neuroendocrine, and musculoskeletal status as well as other organ systems should be considered. The effects of each GFP are pluripotent and do not occur in isolation but rather in concert one with another as well as with other gut

and systemic hormones, cytokines, and adipokines. Overall, the effects of the GFPs are determined by and reflect the complex interplay of a broad spectrum of integrated physiological mechanisms (including orexigenic and anorexigenic hormones, peptides, and amines synthesised in the gut, brain, and adipose tissue) responsible for survival, and, therefore, the effects of GFPs should be studied as a whole. It is more appropriate to interpret each of the pleiotropic actions of GFPs – additive, synergistic, or counter-regulatory responses – as components of a highly coordinated homeostatic network [26, 91, 162, 175, 210, 244, 255, 376, 405, 423, 426, 427, 429, 431, 443]; an extensive body of literature delineates bi- and multi-directional effects between GFPs, as well as between GFPs and other hormones and cytokines (e.g., ghrelin–insulin, ghrelin–glucagon, ghrelin–leptin, etc.), effects which are both site specific and age/gender dependent.

The specific tissue/organ effects of GFPs depend on: (1) peptides production and release, (2) GOAT activity, (3) cellular and serum levels, (4) cell receptors status, (5) endocrine and/or autocrine/paracrine actions, and (6) interactions with other regulatory molecules and pathways.

Particular attention should be given to the fact that the GFPs play critical roles in the regulation of different biological processes through a multitude of signalling and metabolic pathways. At the cellular level, the pluripotent actions of GFPs are mediated by affecting apoptosis, autophagy, oxidative, immune, inflammatory, and fibrotic responses. Through these tightly interacting mechanisms, GFPs participate in maintaining cellular, tissue, and organism integrity by controlling cell differentiation, proliferation, metabolism, and survival in different organ systems. The multiple and diverse roles of GFPs in promoting overall health and longevity indicate their relevance in the pathogenesis of OP/OFs.

This brief overview on the complex biochemical and physiological characteristics of four GFPs, the key regulators of energy balance, which simultaneously synergistically and/or reciprocally exert pluripotent metabolic effects on different tissues and are involved in the regulation of most organ systems, should serve as the basis for understanding their roles in regulating the musculoskeletal system in health and disease.

Table 1. Summary of physiological effects of ghrelin gene peptides (ghrelin, obestatin, and nesfatin-1)

Organ systems and functions	Acyl ghrelin [AG]/total ghrelin	Des-acyl ghrelin [DAG]	Obestatin	Nesfatin-1
Musculoskeletal system				
Osteoblast proliferation and differentiation	↑	↑	↑	↑
Osteoclast differentiation	↓, ↑ (in young age)			↓
Osteocytes				
Chondrocyte differentiation	↑			↑
Bone mineral density	↑			
Myoblast differentiation and fusion	↑	↑		
CNS and complex actions				
Appetite/food intake	↑[75, 117, 146, 148, 233, 238, 398, 399, 403, 409, 444-453] =[331, 454, 455]	↓[181, 182, 369, 422, 443, 456-460] =[452]	↓[126, 199, 255, 391, 413, 422, 461-471], =[365, 393, 394, 472-485], ?[118, 119, 392, 396, 486, 487]	↓[20, 68, 206, 212, 218, 231, 251, 371, 377, 488-500] =[501, 502]
Drinking behaviour	↓[503-505]		↓[391]; =[482]	↓[506]
Energy expenditure, metabolism, thermogenesis	↓[42, 92, 155, 238, 337, 397, 431, 448, 452, 507-516]	↑[435, 456, 517]	=[394, 485]	↑[68, 495, 518-523]

Behaviour, learning, memory, motivation	↑[42, 385, 386, 388, 409, 524-539]	↑[460, 540, 541] ↓[542]	↑[543, 544]	↓[69, 212, 545-556]
Wake/sleep rhythm (wakefulness)	↓[532, 557-563]		↓[564, 565]	↑[212, 566, 567]
Autonomic nervous system SNS	↓[234, 273-275, 402, 507, 568-577]			↑[374, 493, 578-581]
PNS Vagal afferent activity	↓[280, 574, 582-584] ↓[241, 277, 279, 409, 413, 585-588]	=[413]	↓[411, 413]	↑[589-592]
Endocrine system				
Growth hormone	↑[18, 42, 75, 90, 271, 447, 593-599]	=[175]	↓[600]	↓[72, 278]
Hypothalamic–pituitary–adrenal axis	↑[90, 145, 459, 539, 596, 601-603]	=[145, 175, 459]	↑[600]; =[199, 393, 394]	↑[604, 605]
Hypothalamic–pituitary–thyroid axis	↑[606-608] ↓[93, 609, 610]; =[611]	↑[612] ↓[613, 614]	↑[607, 610, 615, 616] =[393]	↓[617, 618]
Hypothalamic–pituitary–gonadal axis	↑[42, 90, 114, 144, 598, 612, 613, 619-639]; =[640]	↓	↑ [641, 642] ↓ [614]; =[393, 640]	↓[205, 496, 617, 643-651] =[652]
Digestive system				
Stomach				
Acid and pepsin secretion	↑[409, 472, 653-662]	=[472, 656]		↓ [204, 663]
Hormones: Gastrin Somatostatin	↑[30] ↓[664], ↑[665]			

Motility	↑[21, 118, 256, 400, 412, 413, 422, 425, 653, 658, 666-682]	↓[412, 413, 425, 456, 457, 658, 673]	↓[396, 411-413, 425, 471, 658, 673]; =[124, 393, 476, 481, 683, 684]	↓[251, 556, 663, 678, 685-690]
Small intestine				
Motility	↑[118, 666, 668, 669, 671, 674, 677, 680, 691-694]		↓ [396, 471, 695] ↑[696, 697]	↓[556, 689]
Hormones: CCK GLP-1 PYY	 ↑[698], ↓[699] ↑[700-702], ↓[699] ↓[699]			 ↑[703] ↑ [704] ↓[703]
Pancreas				
Enzymes	↑[698, 705-707], ↓[708]			
Insulin	↓[42, 86, 146, 148, 175, 271, 285, 286, 419, 709-720]; ↑[30, 721]	↑[175, 352] =[722]	↑[185, 396, 471, 723-726]	↑[218, 496, 518, 727-734]
Glucagon	↑?[271, 711, 735, 736] =[177, 669, 719]			↑[727]
Somatostatin and PP	↑[662, 665, 737]			
Liver				
Gluconeogenesis	↑[271, 433, 452, 738]	↓[433]		↓[739]
Lipogenesis	↑[515, 740-743]	↑[180], ?[513, 744]		↓[581, 745]
Colon				
Motility	↑[746]			
Gut microbiota interaction	↑[351, 747-749]			=[750]

Cardio-vascular system				
Differentiation of cardiomyocytes, endothelial cells, fibroblasts	↑[577, 751-757] ?↓[758, 759]	↑[359, 760]	=[761]	[762-764]
Cardiac output, contractility	↑[42, 88, 146, 570, 576, 765-782]; ↓[783, 784]	↑[785, 786]; =[765] ↓[417]	↑ [255, 775, 787-789]	↓[590, 648, 790-793] ↑[794-796]
Heart rate	↓[574, 772, 797], =[798]	↓[797]		↓[589, 791], ↑[799]
Blood pressure	↓[573, 574, 691, 756, 771, 777, 778, 781, 797, 798, 800-809]	↓[797, 806, 810-816] =[765, 809]	↓[255, 775, 813, 817-819] =[820, 821]	↑[492, 493, 578-580, 604, 764, 790, 799, 822-825]
Renal functions	↑[826-830]			↑ [831]
Lung functions	=[832]; ↑[833]		=[832]	↑[834, 835]
Immune system				
Innate				
Anti-inflammatory macrophages (M2)	↑[836]	↑[837]		=[838]
Pro-inflammatory macrophages (M1)	↓[836, 837, 839]	↓ [837]		=[838]
Neutrophils	↓[**[840]**			↓[835]
Adaptive				
Th1 and Th17	↓[839]			↑ [841]
Th2 and regulatory T cells	↑[842]			

Adipose tissue				
Fat mass/lipogenesis	↑[148, 349, 397, 437, 452, 510, 515, 843-848]	↓[137, 183, 435, 456, 628, 849] ↑ [180, 363, 843]	↓[126, 255, 467, 469, 471] =[394, 480, 483-485]	↓[64, 252, 371, 495, 733] ↑[215, 850]; =[838, 851, 852]
Cellular and molecular actions				
Apoptosis	↓[80, 87, 88, 355, 357, 360, 436, 807, 853-874]	↓[87, 351, 355, 357, 360, 436, 857, 863, 875]	↓[80, 255, 485, 487, 787] =[761]	↓[876-887] ↑[880]
Autophagy	↑[155, 863-866, 888, 889]	↑[890]		↓[882, 886, 891]
Reactive oxygen species (ROS) generation	↓[813, 855, 892-902]	↓[351, 760, 785, 816, 855, 895, 903-906]	↓[80, 544, 813]	↓[878, 879, 882, 883, 886, 887, 907, 908]
Production of NO	↑[155, 472, 777, 804, 813, 815]	↑[813]; =[816]	↑[789, 813, 817]	↓[825]
Regulation of miRs	↑[755, 757, 870, 909-913]	↑[760]		
Mammalian target of rapamycin (mTOR) signalling pathway				↑[414, 416, 739, 908, 914]
Inflammatory response Pro-inflammatory cytokines (TNF-α, INF-γ, IL-1α, IL-1β, IL-6, IL-12, IL-15, IL-17, and IL-18)	↓[80, 91, 568, 584, 586, 587, 833, 836, 839, 842, 864, 892, 902, 915-952]	↓[905]	↓[80, 255, 396, 953] ↓[954]	↓[835, 838, 876, 877, 881, 883-885, 887, 955-957] ↑[217]