Ghrelin gene products, receptors, and GOAT enzyme: biological and pathophysiological insight

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Abstract

Ghrelin is a 28-amino acid acylated hormone, highly expressed in the stomach, which binds to its cognate receptor (GHSR1a) to regulate a plethora of relevant biological processes, including food intake, energy balance, hormonal secretions, learning, inflammation, etc. However, ghrelin is, in fact, the most notorious component of a complex, intricate regulatory system comprised of a growing number of alternative peptides (e.g. obestatin, unacylated ghrelin, and In1-ghrelin, etc.), known (GHSRs) and, necessarily unknown receptors, as well as modifying enzymes (e.g. ghrelin-*O*-acyl-transferase), which interact among them as well as with other regulatory systems in order to tightly modulate key (patho)-physiological processes. This multiplicity of functions and versatility of the ghrelin system arise from a dual, genetic and functional, complexity. Importantly, a growing body of evidence suggests that dysregulation in some of the components of the ghrelin system can lead to or influence the development and/or progression of highly concerning pathologies such as endocrine-related tumors, inflammatory/cardiovascular diseases, and neurodegeneration, wherein these altered components could be used as diagnostic, prognostic, or therapeutic targets. In this context, the aim of this review is to integrate and comprehensively analyze the multiple components and functions of the ghrelin system described to date in order to define and understand its biological and (patho)-physiological significance.

Introduction

Ghrelin is an acylated hormone identified by reverse pharmacology as a 28-amino acid peptide highly expressed in rat stomach (Kojima et al. 1999). Specifically, the ghrelin receptor (GHSR) was first discovered by its ability to bind artificial compounds with growth hormone (GH)-releasing activity (hexarelin or GHRP6) (Howard et al. 1996) and, subsequently, ghrelin was identified as the endogenous ligand for this orphan receptor (Kojima et al. 1999). Intriguingly, evidence accumulated during the last 15 years has revealed that the ghrelin system is an extraordinarily sophisticated and multifunctional regulatory system composed of a number of related peptides and receptors present in a wide variety of tissues where they can exert endocrine, paracrine, and autocrine actions

(Nishi *et al*. 2011). The multiplicity and versatility of the ghrelin system resides in a dual, genetic and functional complexity. From a genetic point of view, the originally identified ghrelin peptide is one of the components of a growing family of related peptides generated by processes of alternative splicing of the ghrelin gene or by post-transcriptional modifications (Seim *et al*. 2009). From a functional point of view, the ghrelin system has been found to be involved in the modulation of a multiplicity of (patho)-physiological functions such as hormonal secretions, memory and learning processes, food intake, body weight gain, insulin release, β-cell survival, adiposity, energy homeostasis, inflammatory processes, as well as in the development and progression of several types of cancers.

In this context, the aim of this review is to integrate and comprehensively analyze the multiple components and functions of the ghrelin system in order to define and understand its biological and (patho)-physiological significance. Particularly, certain endocrine and non-endocrine actions attributed to the ghrelin system are discussed in this review with the purpose of highlighting its dual, genetic and functional, complexity.

Molecular biology of ghrelin system components
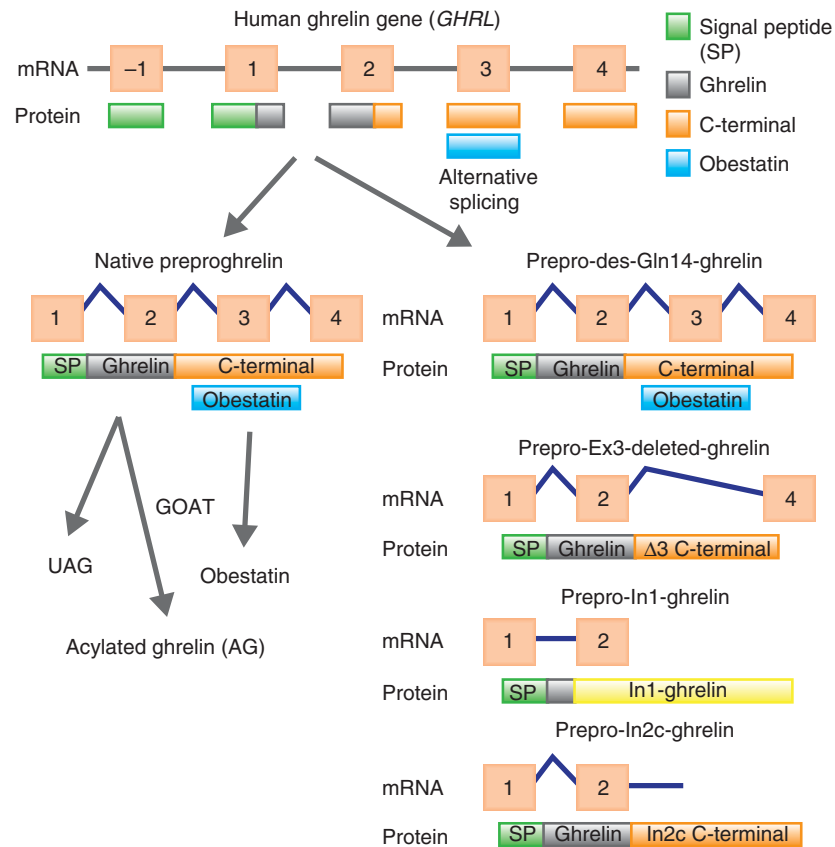
Ghrelin gene (GHRL)

The initially identified ghrelin peptide results from proteolytic processing of a precursor peptide named preproghrelin. In humans, preproghrelin is encoded by a single-copy gene (*GHRL*) located on the short arm of chromosome 3, which was originally thought to be composed of four coding exons (exons 1–4) (Sato *et al*. 2012). However, more recent studies have revealed the existence of a number of alternative upstream exons in the ghrelin gene (exon -1, exon 0, and extended exon 1) that can act as alternative sites for transcription initiation, which considerably increases the complexity of this gene (Seim *et al*. 2009).

The originally identified preproghrelin mRNA transcript encodes a 117-amino acid long peptide in humans, whose sequence is highly conserved among the orthologous mammalian ghrelin prepropeptides (Seim *et al*. 2009, Sato *et al*. 2012). Specifically, human preproghrelin contains a signal peptide (23-amino acids) and a 94-amino acid segment called proghrelin, which undergoes a proteolytic processing that leads to the generation of the mature native ghrelin peptide, and also, to an additional

C-terminal peptide named C-ghrelin (Sato *et al*. 2012; Fig. 1). C-ghrelin peptide can subsequently undergo further proteolytic processes generating an alternative, functional peptide named obestatin, which was initially thought to be the antagonist hormone for ghrelin (Zhang *et al*. 2005; Fig. 1). Therefore, the originally identified native pre-proghrelin mRNA transcript yields, at least, two functional peptides, whose relevance during evolution is supported by the fact that the sequences of both hormones are highly conserved among vertebrates, and particularly, among mammalian genes (Kaiya *et al*. 2008).

In addition, native ghrelin peptide is subjected to a unique modification consisting of the acylation (addition of an octanoyl group) of the third serine residue (Ser3), which is well conserved among mammalian species (Nishi *et al*. 2011). According to its acylation status, the peptide can be termed unacylated ghrelin (UAG) or acylated-ghrelin (AG), AG being the initially identified peptide (Kojima *et al*. 1999), which corresponds to the active peptide able to bind its canonical receptor, GHSR1a. The enzyme responsible for ghrelin acylation, known as ghrelin-*O*-acyl-transferase or GOAT, was discovered simultaneously by two laboratories (Gutierrez *et al*. 2008, Yang *et al*. 2008*b*; Figs 1 and 2). The acylation process by GOAT has been proposed to occur after signal peptide cleavage and before proteolytic processing (Garg 2007; Fig. 2); however, to date, it is not clear whether UAG is the product of an incomplete acylation of the nascent ghrelin peptide, or results from deacylation of mature AG (Nishi *et al*. 2011). Strikingly, although UAG represents over 90% of total blood ghrelin (Patterson *et al*. 2005), it does not bind the classic ghrelin receptor (GHSR1a), and its biological functions are not yet fully elucidated (Kojima *et al*. 1999).

Soon after the discovery of native ghrelin, several independent laboratories have identified a number of alternative ghrelin gene-derived peptides and mRNA splice variants (Seim *et al*. 2009, Gahete *et al*. 2011*a*, Nishi *et al*. 2011; Fig. 1). Some of those mRNA splicing variants generate peptides with small changes in their sequences compared with native ghrelin. This is the case of des-Gln14-ghrelin, which is identical to native ghrelin except for the deletion of one glutamine residue (Gln14). Unfortunately, the differences in function between this 27-amino acid peptide hormone and native ghrelin peptide are unknown (Sato *et al*. 2012). However, the ghrelin gene can also undergo additional processes of alternative splicing such as exon skipping or intron retention. Specifically, an event of exon 3 skipping has been documented (Yeh *et al*. 2005), which generates

Figure 1

Overview of the human ghrelin gene structure and functionally relevant ghrelin gene derived variants (transcripts and putative peptides). Signal peptides are shown in green, ghrelin in grey, obestatin in blue and C-peptides in orange. In the case of the In1-ghrelin variant, the sequence that is shared with ghrelin (exon 1; 12-amino acids) is shown in grey and the unique sequence generated by the retention of intron 1 is shown in yellow.

a 91-amino acid peptide named the Ex3-deleted ghrelin that lacks the coding region for obestatin (Fig. 1). Although the precise functions of the Ex3-deleted ghrelin variant remain uncertain, its expression is increased in human prostate and breast cancers (Seim *et al*. 2009), suggesting a putative role in these pathologies. In addition, a novel ghrelin variant generated by retention of intron 1 (In1), and consequently named In1-ghrelin, has been recently identified. Similar to that found for Ex3-deleted ghrelin, In1-ghrelin variant is overexpressed in breast cancer samples (Gahete *et al*. 2011*a*). Interestingly, In1-ghrelin variant shares the signal peptide and initial portion of the peptide with native ghrelin, including the first 5-amino acids, which is the minimum sequence required for ghrelin acylation by GOAT and for binding and activation of GHSR1a (Seim *et al*. 2009); however, the amino acid sequence of In1-ghrelin is subsequently altered by the retention of In1 (Fig. 1). Interestingly, similar intron retention processes have been identified in other mammalian species (Kineman *et al*. 2007,

Gahete *et al*. 2011*a*). Specifically, the orthologous counterparts of the In1-ghrelin variant have also been found in mice (named In2-ghrelin (Kineman *et al*. 2007)) and in a nonhuman primate model (Gahete *et al*. 2011*a*), which suggest that this new variant might exert an important physiological role that is conserved among vertebrates. Of note, similar to that observed for native preproghrelin, In1-ghrelin prepropeptide also exhibits sites for putative protease action, suggesting that the full peptide could also be processed to yield mature peptides.

Ghrelin receptor gene

The GHSR is encoded by a conserved single-copy gene (*GHSR*) located on chromosome 3 in humans (Camiña 2006). Human GHSR gene is composed of two exons, whose alternative splicing can generate two mRNA species, named GHSR1a and GHSR1b. GHSR1a mRNA, which includes exons 1 and 2, encodes a 366-amino acid G protein-coupled receptor (GPCR) with seven
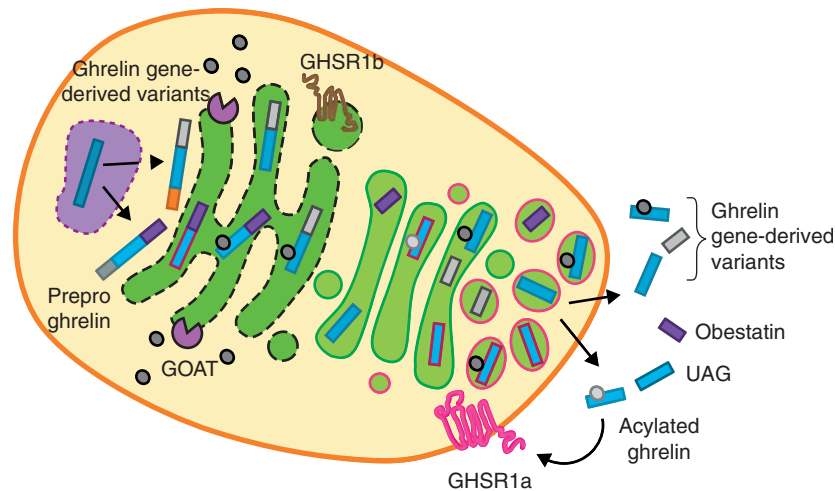
Figure 2

Overview of the post-transcriptional processing of native ghrelin and ghrelin gene derived peptides. During the post-translational modification of the native pre-pro-ghrelin, Ser3 can be acylated by GOAT. This modification of ghrelin is essential for binding to its only known receptor, GHSR1a, and to exert the majority of its biological functions. There is also a truncated orphan variant of the receptor, GHSR1b, with an unknown ligand and function. Additional ghrelin gene derived variants are likely to be similarly processed, generating a number of putative functional peptides, whose target receptors and biological actions are still to be elucidated.

transmembrane domains (TMDs). In contrast, GHSR1b mRNA results from retention of the intron located between exons 1 and 2, which generates a 289-amino acid GPCR isoform with only five TMDs, and having a dissimilar 24-amino acid sequence at the C-terminal region compared with the GHSR1a sequence. To date, the functional activity of truncated GHSR1b remains to be fully elucidated (Camiña 2006), while it is well-established that GHSR1a is the receptor responsible for transducing the signal of AG and the family of synthetic GH secretagogues (GHSs; Cong *et al*. 2010). In fact, the interaction of GHSR1a and AG is fundamentally determined by the high conformational flexibility introduced by ghrelin when it is modified with Ser3 acylation. Particularly, experimental acylation of ghrelin peptide at other Ser residues (Ser2, 6, and 18) has been found to reduce ghrelin functionality (Bednarek *et al*. 2000). Thus, the N-terminal region of Ser3 acyl-ghrelin might bind to the transmembrane cores of the GHSR1a, determining a particular orientation of the molecule where the C-terminal region of AG interacts with the exoloops and/or with the N-terminal segment of the receptor (Camiña 2006).

Additional receptors for ghrelin gene-derived peptides

The family of receptors that mediate the actions of ghrelin gene-derived peptides is yet to be fully identified.

The putative presence of specific uncharacterized GHSR(s), different from GHSR1a, has been previously suggested in chondrocytes, human erythroleukemic HEL cells, and cardiomyocytes (Camiña 2006). In addition, the existence of a common uncharacterized receptor that mediates the biological effects shared by AG and UAG has also been postulated in breast cancer cell lines, cardiomyocytes, human prostatic tumors, and related cell lines (Cassoni *et al*. 2004). Also, the receptor(s) for obestatin is/are still undetermined. Although this peptide was initially reported to bind and activate the orphan G-protein-coupled receptor 39 (GPR39; Zhang *et al*. 2005), this notion is still a matter of debate and is losing support (Lauwers *et al*. 2006, Bassil *et al*. 2007, Chartrel *et al*. 2007); more recently, the glucagon-like peptide-1 receptor (GLP-1R) has been postulated as an alternative receptor for obestatin (Granata *et al*. 2008, 2012). Similarly, the receptor(s) that mediate(s) the biological actions of the recently identified splicing variants (In1-ghrelin, Ex3-deleted ghrelin, etc.) is/are still unknown.

Ghrelin-*O*-acyl-transferase

As mentioned above, ghrelin can be post-translationally modified at its Ser3 by GOAT (Gutierrez *et al*. 2008, Yang *et al*. 2008b), an enzyme that belongs to the superfamily of membrane bound *O*-acyltransferases (MBOATs; Chang & Magee 2009), which is also named MBOAT4

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(Lim *et al.* 2011*b*). The human GOAT gene (*MBOAT4*) is located on chromosome 8p12 and its sequence is highly conserved across vertebrates (Muller *et al.* 2011). Indeed, amino acid sequences of human, mouse, and rat GOAT proteins show a 90% identity, and mouse and rat GOAT enzymes can faithfully octanoylate human ghrelin (Muller *et al.* 2011). Specifically, GOAT is a polytopic integral membrane protein that octanoylates Ser3 of proghrelin in the endoplasmic reticulum (ER) lumen after signal peptide cleavage (Taylor *et al.* 2013). GOAT enzyme is able to process a range of fatty acids, being the most likely acyl donors for the acyl-coAs and, for this reason, GOAT has been postulated to be a putative transporter of acyl-coA from the cytosol to the ER lumen (Yang *et al.* 2008*b*). GOAT mRNA is widely expressed in human tissues such as stomach, pancreas, skeletal muscle, heart, intestine, and bone (Lim *et al.* 2011*a*), a tissue expression pattern that partially resembles that exhibited by ghrelin (Lim *et al.* 2011*a*). Indeed, GOAT protein and mRNA transcripts exist in individual ghrelin-containing cells as is the case of gastric mucosal oxyntic cells (Sakata *et al.* 2009). More-over, some studies have shown that GOAT mRNA expression levels do not exactly correlate with ghrelin expression levels, but they parallel the expression levels of the In1-ghrelin variant (Gahete *et al.* 2011*a*), suggesting the existence of putative additional targets for GOAT.

Physiological role of the ghrelin system

As previously indicated, the ghrelin system has been found to be involved in the modulation of a wide array of physiological functions. Specifically, the ghrelin system is commonly known by its ability to regulate not only endocrine actions (i.e., controlling pituitary hormone or pancreas secretions) but also some non-endocrine actions such as memory and learning processes (Gahete *et al.* 2011*b*), food intake (Nakazato *et al.* 2001), body weight gain, β-cell survival, adiposity, or energy homeostasis (Kirchner *et al.* 2012).

Endocrine actions

Pituitary gland The pituitary gland represents the main target for the endocrine actions of the ghrelin system. In fact, the GHSR was originally identified from swine, human, and rat pituitary glands (Howard *et al.* 1996, Guan *et al.* 1997, Korbonits *et al.* 1998). Accordingly, AG produced in the stomach was initially thought to regulate pituitary function in an endocrine manner. However, it has been demonstrated that native ghrelin is also expressed at the

pituitary gland (Korbonits *et al.* 2001), suggesting the existence of a paracrine and/or autocrine regulation by locally produced ghrelin (Luque *et al.* 2006, Luque & Kineman 2007). This idea is further reinforced by the fact that GOAT is also expressed in the hypothalamus and pituitary gland (Gahete *et al.* 2010*a*, Lim *et al.* 2011*a*). In addition, alternative ghrelin gene-derived variants, such as human In1-ghrelin or murine In2-ghrelin, have been also found to be present and regulated under different physio-logical conditions in the pituitary gland (Kineman *et al.* 2007, Gahete *et al.* 2011*a*), which further supports the idea of the existence of a complex regulation of pituitary function by the ghrelin system. In this sense, although AG was initially identified as a GH-secretagogue, it is nowadays well established that AG also regulates other pituitary secretions from both anterior and posterior pituitary gland.

Growth hormone As mentioned earlier, AG was initially identified by its ability to stimulate GH secretion *in vitro* in several species, including rats (Kojima *et al.* 1999, Tolle *et al.* 2001), mice (Luque, Córdoba-Chacón, Ibáñez-Costa, Gesmundo, Grande, Gracia-Navarro, Tena-Sempere, Ghigo, Gahete, Granata, Kineman and Castaño, 2013, unpublished observations), pigs (Glavaski-Joksimovic *et al.* 2003, Malagon *et al.* 2003), goldfish (Grey & Chang 2011, 2013*a*,*b*), and baboons (Kineman & Luque 2007), where it was demonstrated that AG is as potent as GHRH, the classical GH-releasing factor, in the stimulation of GH secretion (Kineman & Luque 2007). In addition, the crucial role of native AG in the control of GH secretion has been extensively demon-strated *in vivo*, where AG potently stimulates GH release in humans (Takaya *et al.* 2000, Arvat *et al.* 2001, Hataya *et al.* 2001, Nagaya *et al.* 2001, Tassone *et al.* 2003, Garin *et al.* 2013) and rodents (Fernandez-Fernandez *et al.* 2005). Indeed, AG has been shown to be directly involved in the generation of the pulsatile GH secretion pattern (Nass *et al.* 2008), in that GH pulses and AG concentrations are highly correlated in a 1-hour interval during and before the pulse in healthy human subjects. AG seems to act through a variety of mechanisms and to establish a bidirectional, intricate crosstalk with other GH modu-lators in order to finely regulate GH-secretion. Specifically, it has been indicated that peripheral (stomach) AG could act via vagus nerve to increase GHRH and neuropeptide Y (NPY) release, while centrally (hypothalamus) injected AG stimulates GHRH secretion and antagonizes somatostatin (SST) at the pituitary level (Wagner *et al.* 2009, Feng *et al.* 2011). Furthermore, SST inhibits not only GH but also

ghrelin release in healthy subjects, GH-deficient subjects, and acromegalic patients, in whom SST inhibited ghrelin plasma levels independently of GH status (Norrelund *et al.* 2002). The above-mentioned actions of AG seem to be mainly mediated by its cognate receptor GHSR1a in that GH expression is significantly decreased in GHSR-knock-out (KO) mice compared with WT mice (Yang *et al.* 2008*a*).

In contrast to the well-established effect of native AG on the modulation of GH secretion, the role of other ghrelin gene-derived variants is still to be fully characterized. Specifically, to date, no effect of UAG has been found in the modulation of GH production/secretion (Kojima *et al.* 1999, Muccioli *et al.* 2002). In addition, the role exerted by obestatin on GH secretion is controversial. Thus, although some studies did not observe any effect of obestatin on GH secretion (Zhang *et al.* 2005, Nogueiras *et al.* 2007), others found a positive role for this peptide (Pazos *et al.* 2009). However, to the best of our knowledge, all studies performed to determine the role of obestatin on GH secretion have mainly used rat pituitaries or rat tumor cell lines and, therefore, the information gathered till now is limited and fragmentary. Interestingly, unpublished observations from our group indicate that obestatin inhibits GH expression and release in mouse and primate primary pituitary cell cultures, actions that are also observed *in vivo* in mice treated with obestatin (Luque, Córdoba-Chacón, Ibáñez-Costa, Gesmundo, Grande, Gracia-Navarro, Tena-Sempere, Ghigo, Gahete, Granata, Kineman and Castaño, 2013, unpublished observations).

Prolactin   Native AG has also been found to stimulate prolactin (PRL) release from lactotrope cells (Arvat *et al.* 2001, Broglio *et al.* 2003, Tassone *et al.* 2003, Garin *et al.* 2013). This stimulatory effect has been shown to be independent of the well-known stimulator of PRL release, thyrotropin-releasing hormone (Messini *et al.* 2010*b*); however, it depends on the dopaminergic input. Specifically, AG-induced PRL release is not enhanced by a dopamine receptor blocker (Messini *et al.* 2011), but its effect is completely blocked by dopamine agonists (Messini *et al.* 2010*a*), suggesting a functional crosstalk between ghrelin and the dopamine systems. However, the molecular bases underlying this interaction are still to be elucidated. Interestingly, recent studies have suggested that the GHSR, GHSR1a, is also able to mediate AG-independent PRL stimulation in response to other ligands (Cordoba-Chacon *et al.* 2011). Specifically, cortistatin but not its related peptide, SST, is able to increase PRL secretion in mouse and baboon primary pituitary cell cultures, an effect that was completely blocked after a preincubation with a specific

antagonist of the GHSR1a (Cordoba-Chacon *et al.* 2011). On the other hand, neither i.v. nor i.c.v. administration of obestatin has been shown to affect PRL secretion in rats (Yamamoto *et al.* 2007), while the effect of other ghrelin gene-derived variants has not been studied to date.

Proopiomelanocortin   Proopiomelanocortin (POMC) is a precursor polypeptide with 241-amino acids mainly synthesized in corticotroph cells of the anterior pituitary gland, whose cleavage processing leads to the production of adrenocorticotrophin hormone (ACTH). Native AG (Arvat *et al.* 2001, Broglio *et al.* 2003, Tassone *et al.* 2003, Vestergaard *et al.* 2007, Milosevic *et al.* 2013) or the synthetic GHSs (Korbonits *et al.* 1995, 1999, Bowers & Granda-Ayala 1996, Arvat *et al.* 1997, Clark *et al.* 1997, Thomas *et al.* 1997) have been shown to stimulate ACTH secretion in several species, such as humans and rats. However, this effect could depend on the metabolic/ energy status, as AG-induced ACTH secretion has been shown to be impaired in obesity (Milosevic *et al.* 2013). Interestingly, recent studies have suggested that AG could be controlling ACTH release by acting at several levels, in that AG can act directly at the pituitary level by increasing free cytosolic calcium levels (Yamazaki *et al.* 2012) and also at the hypothalamic level by stimulating corticotrophin-releasing hormone (CRH) and arginine–vasopressin (AVP) production in rats (Wren *et al.* 2002). Although other ghrelin gene-derived products such as UAG and obestatin do not seem to exert any significant effect on the modulation of ACTH production and/or secretion (Yamamoto *et al.* 2007), the ghrelin gene plays a crucial role in the regulation of the stress response. Indeed, ACTH levels were significantly lower in ghrelin KO mice in response to stress, suggesting that some endogenous ghrelin gene-derived products, probably AG, modulate the response to acute stress, controlling the hypothalamic–pituitary–axis and diminishing anxious behavior under stress (Spencer *et al.* 2012).

Other anterior pituitary hormones   The ghrelin system has also been shown to exert a crucial role in the regulation of other anterior pituitary hormones, through direct or indirect actions. Specifically, AG administration was shown to inhibit LH release in rats (Furuta *et al.* 2001, Fernandez-Fernandez *et al.* 2005), and FSH and LH in healthy men (Kluge *et al.* 2007, Lanfranco *et al.* 2008, Kluge *et al.* 2009) and women (Messini *et al.* 2010*a*, Kluge *et al.* 2012). On the other hand, the role of AG on TSH release is not well defined as some studies indicate a lack of direct effect on TSH release in humans (Takaya *et al.* 2000,

Hataya *et al.* 2001, Nagaya *et al.* 2001), while a recent study indicates that AG administration could induce a decrease in TSH release in healthy subjects (Kluge *et al.* 2010). Given that this study showed that AG was able to increase thyroxine, the authors speculate that the effect of AG on TSH might be mediated by a indirect action at the hypothalamic level through a thyroxine-dependent feedback (Kluge *et al.* 2010). Finally, glycoprotein hormones α chain (CGA) is the common subunit for FSH, LH, and TSH (and chorionic gonadotropin hormones) and is normally released as part of a heterodimer with these β subunits. Interestingly, despite the lack of studies addressing the ability of the ghrelin system to modify CGA release, a recent work has demonstrated, by double immunostaining, that ghrelin is present in the majority of α-subunit-producing cells (Rotondo *et al.* 2012), suggesting a possible functional role in its biogenesis.

Posterior pituitary hormones   The ghrelin system has also been found to regulate the secretion of posterior pituitary hormones, including AVP and oxytocin (OT); however, this could be an indirect effect as studies in transgenic mice in which GHSR was labeled with GFP indicate that GHSR is not present in posterior pituitary (Reichenbach *et al.* 2012). Thus, native AG stimulates AVP release from rat posterior pituitary gland, probably via an indirect action mediated by NPY neurons (Ishizaki *et al.* 2002, Wren *et al.* 2002). Notably, AG-induced ACTH release has also been suggested to be mediated by the AG action on AVP secretion, because the AG-induced AVP rise precedes the increase in ACTH and cortisol levels in response to AG injection in healthy men (Coiro *et al.* 2005). On the other hand, the relationship between the OT and ghrelin systems has not been fully elucidated. While it has been demonstrated that OT could not inhibit the stimulatory effect of AG on GH, PRL, and ACTH release in healthy subjects (Coiro *et al.* 2011), it might influence the effect of AG on NPY secretion (Coiro *et al.* 2008), which suggests the existence of an intricate relationship between both neuropeptide systems.

Gastrointestinal tract   Ghrelin is mainly produced in the stomach by a specific population of endocrine cells located in the gastric mucosa, the so-called A-like cells (Kojima *et al.* 1999), but it has also been detected in other gastrointestinal tract (GIT) regions such as the small intestine (Ghelardoni *et al.* 2006). Moreover, obestatin is also expressed in the mucosa of the GIT. Indeed, the subcellular localization of obestatin and ghrelin has been shown to be identical, within the same secretory vesicles,

suggesting that obestatin and ghrelin are possibly costored in the same cells in the GIT (Gronberg *et al.* 2008). Similarly, recent studies have also demonstrated that GOAT enzyme expression is highly enriched within gastric ghrelin-producing cells (Sakata *et al.* 2009). Interestingly, other ghrelin gene-derived variants, such as In1-ghrelin, have also been found to be expressed in the GIT (Gahete *et al.* 2011a), although the specific cells where they are produced are yet to be determined. The fact that GHSR expression has also been detected in stomach, as well as in small and large intestine (Gnanapavan *et al.* 2002), suggests that ghrelin may play a role in the regulation of other GIT hormones. Unfortunately, studies reported hitherto have generated conflicting results (Lee *et al.* 2002, Dornonville de la Cour *et al.* 2004, de la Cour *et al.* 2007). Although initial studies suggested that i.v. administration of ghrelin could increase gastrin secretion in rat stomach (Lee *et al.* 2002), later studies did not find any effect of AG or UAG in the secretion of gastrin, SST, or histamine in the same species (Dornonville de la Cour *et al.* 2004). Interestingly, ghrelin secretion is also regulated by other GIT hormones (de la Cour *et al.* 2007). In particular, adrenaline, noradrenaline, endothelin, and secretin seem to stimulate, while SST and GRP seem to inhibit ghrelin secretion at stomach level (de la Cour *et al.* 2007), which suggests the existence of a complex feedback regulatory system among GIT hormone systems.

Pancreas   The endocrine pancreas includes a population of cells (epsilon cells (ε-cells)) that are specialized in the production and secretion of ghrelin (Wierup *et al.* 2002). However, ghrelin production has also been shown in other pancreatic cell types such as α- and β-cells (Date *et al.* 2002, Dezaki *et al.* 2004, Granata *et al.* 2007), suggesting a paracrine/autocrine action of the ghrelin system in the pancreas. Indeed, the endocrine pancreas is a key target tissue for the actions of ghrelin. Specifically, AG plays an important role in the regulation of glucose homeostasis by inhibiting insulin release in mice, rats, and humans (Broglio *et al.* 2001, Egido *et al.* 2002, Reimer *et al.* 2003, Granata *et al.* 2007). AG also modulates the production/secretion of glucagon from the pancreas (Chuang *et al.* 2011) and, indeed, it has been demonstrated that GHSR colocalizes with glucagon and insulin in rat pancreatic islets, which further indicates that the ghrelin system plays an important role in pancreatic α- and β-cells (Kageyama *et al.* 2005). In addition, AG has been shown to be able to modulate insulin actions, as it can induce peripheral insulin resistance (Damjanovic *et al.* 2006, Vestergaard *et al.* 2008) without affecting hepatic

insulin sensitivity (Vestergaard *et al.* 2013). Therefore, due to this crucial role of AG in the regulation of pancreas and insulin physiology, it has been suggested that GHSR blockers and/or GOAT inhibitors might be used as putative antidiabetic drugs in the future (Gualillo *et al.* 2008, Barnett *et al.* 2010).

In addition, obestatin expression has also been shown in the pancreas (Chanoine *et al.* 2006, Zhao *et al.* 2008). However, the role that obestatin may play in the regulation of endocrine pancreas secretions is poorly understood. In fact, obestatin seems to exert dose-dependent and species-specific effects on glucose-induced insulin secretion (Egido *et al.* 2009). At high glucose levels, obestatin inhibits insulin secretion from murine pancreatic islets, while, at low glucose concentrations, it increases insulin secretion from human β cells (Granata *et al.* 2008, Qader *et al.* 2008). Moreover, obestatin probably plays a relevant role in the promotion of human islet cell survival and β-cell functioning (Granata *et al.* 2008). Recent observations have also suggested that other ghrelin gene-derived molecules could be present in the endocrine pancreas, as in the case of the In1-ghrelin variant (Chanclon *et al.* 2013), where it might play a functional role, which merits further investigation.

Adrenal gland   The ghrelin system is known to be involved in the control of the hypothalamus–pituitary–adrenal axis activity in humans and other mammals (Giordano *et al.* 2006). However, the precise role of the ghrelin system in the physiology of the adrenal gland has not been fully elucidated. Specifically, it has been shown that some ghrelin gene-derived hormones, GOAT, and GHSRs are expressed in the cortex and/or the medulla of human and rat adrenal glands (Barreiro *et al.* 2002, Andreis *et al.* 2003, Tortorella *et al.* 2003, Raghay *et al.* 2008, Rucinski *et al.* 2009, Lim *et al.* 2011*a*). Indeed, the GHSR seems to be more abundant in the adrenal zona glomerulosa, the cambium layer involved in the maintenance of adrenal cortex growth (Tortorella *et al.* 2003). Interestingly, ghrelin and GHSR expression seems to be reduced in the human adrenal cortex with aging (Carraro *et al.* 2004, 2006).

Initial reports discarded a direct action of the ghrelin system on adrenal secretion, in that AG and/or obestatin did not significantly affect either basal or agonist (ACTH and angiotensin-II)-stimulated corticosterone secretion from adrenal cells *in vitro* (Barreiro *et al.* 2002, Andreis *et al.* 2003, Tortorella *et al.* 2003, Rucinski *et al.* 2009). However, a more recent study has shown that prolonged *in vitro* exposure to AG, but not to obestatin, could

stimulate corticosterone secretion in rats (Rucinski *et al.* 2009). A more consistent explanation seems to be that AG, via GHSR1a, could be involved in the autocrine-paracrine control of human adrenal growth, as AG can enhance the proliferation rate of cultured zona glomerulosa cells, without affecting the apoptotic deletion rate (Andreis *et al.* 2003). Moreover, AG treatment is able to increase the absolute whole adrenal gland weight and volume in rats *in vivo* (Milosevic *et al.* 2010). Altogether, these data suggest that the ghrelin system could exert a direct role in the autocrine-paracrine regulation of human adrenal growth (Tortorella *et al.* 2003), while it might exert an indirect role, via ACTH release, on glucocorticoid secretion.

Nonendocrine actions

Regulation of energy balance, appetite, and body temperature   AG promotes a positive energy balance by increasing food intake and reducing energy expenditure, therefore leading to an increase in body weight and adiposity (Tschop *et al.* 2000). Indeed, one of the central roles of the ghrelin system is the regulation of feeding and energy homeostasis (Wren *et al.* 2000, Nakazato *et al.* 2001). AG is known as the 'hunger hormone' and several studies indicate that circulating ghrelin levels rise in fasting and between meals, while AG levels decrease during the post-prandial state (Cummings *et al.* 2001). Moreover, AG has been found to stimulate energy intake in experimental animals (Wren *et al.* 2000, Jonsson 2013) and healthy men (Wren *et al.* 2001). Specifically, this hormone exerts its orexigenic effects acting at the level of a brain area known to be crucial in the regulation of feeding: the hypothalamus. Here it activates neurons of the arcuate nucleus, particularly, NPY- and agouti-related peptide (AgRP)-producing neurons (Nazakato *et al.* 2001), probably through the modulation of the mTORC1/S6K1 pathway (Stevanovic *et al.* 2013). AG reaches the hypothalamus through three different pathways: first, through the general circulation, by crossing the blood–brain barrier; secondly, through the vagal afferent nerves; and thirdly, it can be synthesized in the hypothalamus, where it may directly exert paracrine effects (Lim *et al.* 2011*b*). However, the regulation of food intake by AG seems to be tightly dependent on the metabolic status. Recent studies have demonstrated that exogenous AG administration can induce hyperphagia in free-feeding rats but not in animals under negative metabolic conditions, such as fasting or in chronically food-restricted animals (Alen *et al.* 2013). AG has also been shown to increase energy intake in situations of impaired appetite or cachexia, a common problem in

cancer patients (Molfino *et al.* 2013). In striking contrast, UAG has been shown to suppress food intake (Asakawa *et al.* 2005). In that both AG and UAG derive from the same precursor, the inverse effects of these two peptides suggest that the stomach, the main source of circulating AG and UAG, might be involved, somehow, in the sensing and regulation of energy balance. In addition, although obestatin was initially postulated as the ghrelin antagonistic hormone, nowadays its effect on food intake remains strongly controversial. Specifically, obestatin has been shown to suppress food intake and energy balance in some rodent studies (Zizzari *et al.* 2007, Hassouna *et al.* 2012, Gargantini *et al.* 2013), while no effect has been reported by different studies in rats and mice (Gourcerol & Tache 2007, Kobelt *et al.* 2008).

Despite the well-established role of the ghrelin system in the regulation of feeding behavior and energy expenditure, its implication in thermoregulation, an additional process in the regulation of energy balance, is still to be fully elucidated. Initial studies suggested a biphasic action of AG on thermogenesis, in that low doses of AG could reduce body temperature (Lawrence *et al.* 2002), while high doses could increase it (Jaszberenyi *et al.* 2006), probably through a hypothalamic action (Tokizawa *et al.* 2012). This idea is supported by the fact that ghrelin gene KO mice cannot control body temperature under extreme thermic conditions (Szentirmai *et al.* 2009), and because areas of the hypothalamus located in the vicinity of cold-sensitive neurons exhibit ghrelin-binding sites (Wiedmer *et al.* 2011). However, these data are in contrast with other studies showing that, under extreme thermic conditions, both GHSR-KO (Szentirmai *et al.* 2009) and GOAT-KO mice (Heppner *et al.* 2013) can control core body temperature, and with those showing that AG administration does not alter body temperature in mice, rats, and humans (Wiedmer *et al.* 2011). In this scenario, the actions of alternative ghrelin gene-derived peptides could shed some light on this controversy, in that, for example, obestatin has been shown to attenuate the hypothermic response of ghrelin gene KO mice (Szentirmai *et al.* 2009), thereby suggesting a complex regulation of body temperature by the ghrelin system.

Gastric motility and acid secretion AG is a potent accelerator of gastric emptying and a stimulator of GI motility in humans (Tack *et al.* 2006), rats (Trudel *et al.* 2002, Xu *et al.* 2013), and mice (Masuda *et al.* 2000, Fujino *et al.* 2003, Ariga *et al.* 2007, Zheng *et al.* 2009); while this effect was not observed in dogs (Ohno *et al.* 2010). Indeed, gastric emptying is delayed in GHSR-deficient mice

(Yang *et al.* 2013) and, interestingly, these mice exhibited a reduction in the number of nerve cells in the gastric muscle, where GHSR is predominantly expressed (Yang *et al.* 2013). AG induces motor activity in the GIT via a dual, central and peripheral, mechanism (Korbonits *et al.* 2004). The central mechanism is mediated through NPY-neurons of the arcuate nucleus, the vagus nerve, and/or the lateral septum (Gong *et al.* 2013); while peripheral effects include the induction of the GI phase III contractions (Fujitsuka *et al.* 2012). In addition, AG has also been found to be able to stimulate, in a dose-dependent manner, the acid secretion from rat stomach (Masuda *et al.* 2000), probably through a nitric oxide-dependent pathway (Bilgin *et al.* 2008).

Similar to that observed for food intake regulation, UAG has been shown to exhibit an opposite role to that exerted by AG, in that UAG suppresses gastric emptying in the antrum (Asakawa *et al.* 2005). This seems to be an indirect effect mediated via the hypothalamus by the activation of anorexigenic cocaine- and amphetamine-regulated transcripts as well as urocortin gene expression (Asakawa *et al.* 2005). Similarly, obestatin has also been described as an inhibitor of motor activity in the gastroduodenal region in the fed state, but not in the fasted state, an effect that seems to be an indirect action in which CRH type 1 and type 2 receptors might be involved (Ataka *et al.* 2008). However, obestatin was unable to antagonize AG-induced stimulation of gastroduodenal motility (Ataka *et al.* 2008).

Cognitive processes The ghrelin system has been shown to be involved in the regulation of many brain functions such as the modulation of cognitive processes, acting at the hypothalamus and other brain areas. Specifically, i.c.v. application of AG stimulates memory retention (Carlini *et al.* 2002), in a dose-dependent manner (Stoyanova *et al.* 2013) and independent of the brain area injected (hippocampus, amygdala, or dorsal raphe) (Carlini *et al.* 2004), through processes that could include the promotion of neurogenesis (Li *et al.* 2013) and synaptic plasticity (Abizaid *et al.* 2006). Indeed, AG but not UAG can enhance synaptic plasticity through both presynaptic (enhancing presynaptic excitatory inputs) and postsynaptic (elevating excitability of postsynaptic neurons) mechanisms (Chen *et al.* 2011) in the hippocampus, where the AG-induced synaptic changes closely parallel the enhanced hippocampus-dependent spatial learning and memory. Consistently, ghrelin KO mice exhibit impaired performance in behavioral memory testing, which is reversed by AG administration

(Diano *et al.* 2006). The fact that intra-hippocampal AG administration improved long-term memory but did not modify short-term memory (Carlini *et al.* 2010) suggests that AG may modulate memory acquisition/consolidation but not the retrieval. Unfortunately, the role of other ghrelin gene-derived variants in the regulation of cognitive functions is still uncertain. It has been shown that UAG can induce proliferation of neuronal precursor cells in the rat fetal spinal cord (Sato *et al.* 2006); however, the role of obestatin and ghrelin splice variants remains unexplored.

Neuroprotection   Frago *et al.* (2002) provided the first evidence for the neuroprotective effects of ghrelin analogs in several brain areas of adult rats. Further studies have demonstrated the potential of ghrelin to stimulate neural survival in diverse experimental models of ischemia, traumatic brain injury, spinal cord injury, amyotrophic lateral sclerosis, epilepsy, Alzheimer's disease (AD), and Parkinson's disease (PD) (Liu *et al.* 2006, Chung *et al.* 2007, Dos Santos *et al.* 2013, Kenny *et al.* 2013). Of note, the actions of ghrelin in neuroprotection seem to be specifically mediated by the inhibition of proapoptotic molecules associated with mitochondrial pathways and by activating endogenous protective molecules (Miao *et al.* 2007). Interestingly, ghrelin acts by inhibiting apoptotic pathways regardless of its acylation (Chung *et al.* 2008); however, while AG acts through GHSR1a, UAG would bind to the CD36 scavenger receptor to exert these actions (Bulgarelli *et al.* 2009).

Cardiovascular actions   The ghrelin system has also been implicated in the regulation of cardiovascular physiology by acting at different levels of the cardiovascular system (vascular, endothelial, and/or cardiac). Specifically, AG increases cardiac output, decreases blood pressure, increases cardiac contractility and vasodilation, inhibits apoptosis in endothelial cells and cardiomyocytes, and decreases peripheral resistance (Tesauro *et al.* 2010). These actions seem to be mediated by its cognate receptor as the expression of GHSR1a has been found in human cardiomyocytes, myocardium, and aorta (Gnanapavan *et al.* 2002). Similarly, the presence of the truncated GHSR1b variant has also been found in human myocardium; however, its (patho)physiological function, if any, is still unknown (Zhang *et al.* 2010). It is also noteworthy that cardiomyocytes and endothelial cells are able to produce ghrelin itself, suggesting that this peptide could exert paracrine/autocrine actions to regulate cardiac function (Iglesias *et al.* 2004, Kleinz *et al.* 2006).

Interestingly, locally produced UAG has been shown to exert different actions from those observed for AG in terms of the regulation of glucose and fatty acid uptake from cardiomyocytes (Lear *et al.* 2010), which suggests a complex relationship between the ghrelin and cardiovascular systems.

Immune system and inflammation   The ghrelin system has also been reported to be widely distributed among immune- and inflammatory-related tissues and cells. In particular, ghrelin is expressed in a variety of immune cells and tissues such as T-lymphocytes, B-lymphocytes, neutrophils, spleen, or lymph nodes (Hattori *et al.* 2001). The presence of GHSRs in immune cells has also been reported (Hattori *et al.* 2001). Surprisingly, human GHSR1a seems to have a very restricted expression, mainly circumscribed to the spleen, thymocytes, and some subpopulations of immune cells, while the truncated variant GHSR1b has been found to have a wider distribution, including spleen, lymph nodes, thymocytes, lymphocytes, and other peripheral blood mononuclear cells (Hattori *et al.* 2001).

From a physiological point of view, AG has been shown to be able to influence thymopoiesis, as infusion of AG increases the number of thymocytes and thymus size in mice (Dixit *et al.* 2007), suggesting a relevant role of the ghrelin system in the development and maturation of the thymus. Moreover, this is supported by the fact that the accelerated age-dependent involution of the thymus observed in ghrelin KO mice can be partially reversed by AG administration (Dixit *et al.* 2007). Importantly, these results have led to the idea that the decreased immune response observed in the elderly could be improved by enhancing AG signaling. Furthermore, AG can regulate the physiology of the immune system by regulating the production and secretion of a number of proinflammatory cytokines such as TNFα, IL6, IL1β in human and animal models (Hosomi *et al.* 2008), which is further supported by the fact that serum or plasma ghrelin levels are increased in different types of inflammatory diseases including ulcerative colitis, Crohn's disease, ankylosing spondylitis, pancreatitis, colitis, and rheumatoid arthritis (Baatar *et al.* 2011). The presence and the role of other ghrelin gene-derived variants on the immune system remain unknown.

Pathological implications of ghrelin system

Inasmuch as the ghrelin system plays a relevant role in the modulation of a plethora of physiological processes, changes in this regulatory system have also been

associated with the development and/or progression of a number of pathological conditions. Particularly, AG can increase cell proliferation in several healthy and cancer tissues, as well as regulate invasiveness, migration, metastasis, and apoptosis in various cell types (Jeffery et al. 2002, Pettersson et al. 2002, Duxbury et al. 2003, De Vriese et al. 2005, Maccarinelli et al. 2005, Marjchrzak et al. 2012, Yu et al. 2013).

Endocrine-related tumors

Pituitary adenomas Several studies have demonstrated that ghrelin, as well as GHSR1a and GHSR1b, are differentially expressed in pituitary adenomas (Korbonits et al. 1998, 2001, Kim et al. 2001). Specifically, it has been reported that ghrelin expression levels are similar between gonadotropinomas or prolactinomas and normal pituitaries (NPs); however, nonfunctioning pituitary adenomas (NFPA) and somatotropinomas seem to exhibit higher expression levels of ghrelin, while corticotropinomas express lower ghrelin mRNA levels than NPs. On the other hand, while GHSR1a and GHSR1b are poorly expressed in gonadotropinomas, the expression levels of both receptors have been found to be higher in somatotropinomas, corticotropinomas, and prolactinomas compared with NPs but did not differ between NFPA and NPs.

Acromegaly Acromegaly is a syndrome mostly caused by GH hypersecretion from tumoral pituitary somatotrope cells, and subsequent increase in IGF1 production by the liver. The main consequences of chronic exposure to GH and IGF1 are skeletal, tissue, and organ growth, including acral changes, gigantism, arthritis, heart disorders, diabetes, insulin resistance, hypogonadism, etc. (Melmed 2009). As AG is one of the main primary stimulators of the production and secretion of GH, it has been suggested that changes in the ghrelin system might play a role in the development and/or progression of GH-producing adenomas. However, there is controversial data on this topic as circulating levels of ghrelin have been found to be reduced (Cappiello et al. 2002, Freda et al. 2003), elevated (Jaskula et al. 2009), or not changed (Barkan et al. 2003, Jarkovska et al. 2006, Isidro et al. 2007, Jaskula et al. 2009) in acromegalic patients compared with healthy subjects. Interestingly, normalization in circulating ghrelin levels has been observed after adenoma surgery in some (Freda et al. 2003) but not all studies (Kawamata et al. 2007). All these studies have pointed out that factors other than GH, IGFI, and insulin could be differentially influencing the

circulating ghrelin levels in acromegalic patients; as in the case of the lipid profile (Jaskula et al. 2009).

AG enhances GH release in GH-producing adenomas in vitro (Rubinfeld et al. 2004), and has been shown to be locally produced in somatotropinomas (Kim et al. 2001, Korbonits et al. 2001); however, no significant correlation between circulating GH levels before surgery and ghrelin mRNA expression in the tumors has been observed (Korbonits et al. 2001). Surprisingly, a previous study showed that ghrelin mRNA levels in somatotropinomas were negatively correlated with tumor size and, indeed, high-grade tumors had lower levels of ghrelin mRNA than low-grade adenomas (Kim et al. 2001). On the other hand, both GHSR1a and GHSR1b have been found to be highly expressed in somatotropinomas compared with NPs (Barlier et al. 1999, Korbonits et al. 2001, Rubinfeld et al. 2004), which together with the previous data mentioned in this section, might suggest the existence of an autocrine/paracrine role of the ghrelin system in GH-producing adenomas. Moreover, studies in a rat pituitary somatotrope cell line indicate that both AG and UAG can stimulate cell proliferation (Nanzer et al. 2004). In spite of all these studies, the data reported to date which focused on the role that ghrelin plays in GH-producing adenomas are limited and inconclusive; this, together with the fact that the effects of other ghrelin gene-derived variants on GH-producing tumors has not yet been explored, suggest that further work should be conducted to elucidate the precise role of the ghrelin system in the development and progression of GH-producing adenomas.

Prolactinomas Prolactinomas are caused by the presence of PRL-producing (lactotrope) tumoral cells in the pituitary gland, which produces clinical symptoms such as weight gain, infertility, galactorrhea, gonadal dysfunction, headache, hypopituitarism, and visual field defects (Colao 2009). Although the presence of ghrelin and its receptor has been confirmed in prolactinomas (Kim et al. 2001, Korbonits et al. 2001, Rotondo et al. 2011), limited studies have been carried out to elucidate the direct pathological role of the ghrelin system in these tumors. Specifically, a previous study revealed that AG treatment elicited PRL release in prolactinomas and GH–PRL mixed adenoma cell cultures (Rubinfeld et al. 2004), suggesting an involvement of the ghrelin system in the development and progression of prolactinomas. However, to the best of our knowledge, the role of other ghrelin gene-derived peptides, such as UAG or obestatin, on prolactinomas has not been explored so far.

Cushing's disease Cushing's disease is the result of chronic exposure to cortisol due to an ACTH-producing pituitary adenoma and is characterized by central obesity, diabetes mellitus, dyslipidemia, hypertension, hirsutism, muscle weakness, and emotional disturbances (Feelders & Hofland 2013). The expression of some ghrelin gene-derived components and GHSRs has been detected in ACTH-secreting adenomas (Kim *et al.* 2001, Korbonits *et al.* 2001, Martinez-Fuentes *et al.* 2006, Pecori Giraldi *et al.* 2007, Rotondo *et al.* 2011). Indeed, ghrelin, GHSR1a and GHSR1b are highly expressed in ACTH-secreting adenomas, where ghrelin and ACTH have been shown to be costored in the same secretory granules (Martinez-Fuentes *et al.* 2006), suggesting a putative autocrine/paracrine role of the ghrelin system in ACTH-secreting adenomas. Interestingly, it has been indicated that the well-known ACTH releasing effect of AG observed in tumoral corticotrope cells (Pecori Giraldi *et al.* 2007) might be mediated via increased free cytosolic calcium levels (Martinez-Fuentes *et al.* 2006). Finally, a recent study has suggested that AG administration may be used in the diagnosis of Cushing's disease with adrenal enlargement, in that these patients do not respond correctly to a desmopressin test, while they clearly respond to AG, suggesting that AG is able to stimulate ACTH and cortisol release in all forms of Cushing's disease (Miljic *et al.* 2010).

NFPA, gonadotropinomas, and thyrotropinomas Approximately 40–50% of pituitary adenomas are gonadotrope-derived tumors (Chaidarun & Klibanski 2002), most of them being classified as NFPA and null-cell adenomas (Colao *et al.* 2008), which are characterized by the lack of hormone release by the tumor, provoking a variety of symptoms including headache, visual defects, hypogonadism, and hypopituitarism. A small proportion of these gonadotrope-derived tumors secrete FSH and/or LH and are generally named gonadotropinomas (Chaidarun & Klibanski 2002), which may cause ovarian hyperstimulation, testicular enlargement, and precocious puberty. There is also a rare kind of pituitary adenomas that secrete TSH, named thyrotropinomas, characterized not only by the typical pituitary adenoma symptoms (headache, visual defects, and hypopituitarism) but also by thyroid dysfunction (Beck-Peccoz *et al.* 2009). Surprisingly, although the presence of ghrelin system components has been detected in these pituitary adenomas (Kim *et al.* 2001, Korbonits *et al.* 2001, Rotondo *et al.* 2011), no functional studies have been implemented to date and, therefore, the role of the ghrelin system in the pathology of NFPA, gonadotropinomas, and thyrotropinomas is still to be elucidated.

Neuroendocrine tumors Neuroendocrine tumors (NETs) comprise a group of extraordinary heterogeneous neoplasias originated in the diffuse neuroendocrine system of the GIT, where neuroendocrine GIT and pancreas tumors are included. Expression of some components of the ghrelin system has been detected in normal and tumoral tissues of the GIT. Indeed, ghrelin, obestatin, GOAT, and GHSRs are expressed in gastric (Papotti *et al.* 2001, Rindi *et al.* 2002, Volante *et al.* 2002), intestinal (Inhoff *et al.* 2008), and/or pancreatic carcinoids (Volante *et al.* 2002, Ekeblad *et al.* 2007), although their expression levels vary depending on the tissues and the detection method used (Papotti *et al.* 2001, Rindi *et al.* 2002, Volante *et al.* 2002, Inhoff *et al.* 2008). However, it is not clear whether the expression profile of the ghrelin system is altered in NETs compared with control tissues. For example, obestatin seems to be similarly expressed in normal and carcinogenic tissues, but its secretion seems to be reduced in oncological conditions due to post-translational modifications (Volante *et al.* 2002). In addition, most of the studies have shown that plasma ghrelin concentrations lie within the normal range in NETs patients, and ghrelin elevation has been only found in two cases of ghrelinomas. Unfortunately, to the best of our knowledge, the functional role of the ghrelin system components in the development and/or progression of NETs has not been explored to date.

Adrenal tumors The presence and the role of the ghrelin system in adrenal tumors have been largely under-explored. Specifically, some studies have reported variable levels of ghrelin mRNA and/or protein in the majority of several types of human and rat adrenal tumors such as pheochromocytomas, cortisol-secretin adenomas, aldosterone-secreting adenomas, and adrenocortical carcinomas (Barzon *et al.* 2005, Raghay *et al.* 2008, Ueberberg *et al.* 2008). In contrast, expression of the cognate receptor GHSR1a has been undetectable in most of the adrenal tumors analyzed to date. However, its presence has been found in a few pheochromocytomas, cortisol-secretin adenomas, and adrenocortical carcinoma samples (Barzon *et al.* 2005, Ueberberg *et al.* 2008). The presence of other components of the ghrelin system has not been explored and the functional role of the ghrelin system in these tumors is unknown. Nevertheless, the fact that ghrelin expression has been found in various types of adrenal tumors might indicate that this

regulatory system has some unknown pathophysiological functions, which remain to be investigated.

Breast Breast cancer is the most common malignancy in women worldwide and remains a significant health issue in industrialized countries (Siegel *et al*. 2013). Breast cancer seems to be initiated by defined genomic alterations, many of which could be used as therapeutic targets or biomarkers. In line with this, the presence of the majority of the ghrelin system components (ghrelin, obestatin, ghrelin splicing variants, GOAT, and GHSRs) has been extensively proven in normal mammary gland, breast tumors, and breast cancer cell lines (Chopin *et al*. 2012). In particular, native ghrelin and obestatin expression has been found in a high proportion of breast cancer tissues with different grades (Gronberg *et al*. 2012), although its expression does not seem to be increased in breast cancer compared with healthy tissues (Gahete *et al*. 2011a). Indeed, expression of ghrelin and obestatin have been associated with a positive outcome in invasive breast tumor as patients with ghrelin-expressing tumors exhibited 2.5–3 times lower risk for recurrence of breast cancer than those lacking ghrelin expression (Gronberg *et al*. 2012).

However, several ghrelin gene polymorphisms have been associated with an increase in breast cancer risk (Dossus *et al*. 2008), suggesting that a dysbalance in normal expression of the ghrelin system in mammary gland tissue could be involved in breast tumor pathogenesis. This is indeed supported by the observation that several ghrelin gene-derived splicing variants are overexpressed in breast cancer tissue (Jeffery *et al*. 2005, Gahete *et al*. 2010b). In particular, expression of the In1-ghrelin variant is eightfold higher in a series of 40 sporadic invasive ductal breast carcinomas compared with normal breast tissues (Gahete *et al*. 2011a), while Ex3-deleted ghrelin variant is overexpressed in the MDA-MB-435 metastatic breast cancer cell line relative to the benign MCF-10A breast epithelial cell line (Jeffery *et al*. 2005). In addition, GOAT expression has recently been described in breast cancer cell lines, and its expression seems to be elevated in breast cancer tissues compared with normal breast samples (Gahete *et al*. 2011a). Interestingly, GHSR1a is also present in both normal and tumoral breast samples (Jeffery *et al*. 2005, Gahete *et al*. 2011a), while the GHSR1b receptor seems to be selectively expressed in breast carcinoma, where its abundance shows a strong correlation with In1-ghrelin expression levels (Gahete *et al*. 2011a). Indeed, expression of the cognate GHSR GHSR1a could even be reduced in

breast cancer samples compared with normal mammary gland (Gahete *et al*. 2011a). In line with this, it has been suggested that the hypermethylation of the GHSR promoter region could be the underlying cause of this phenomenon, as hypermethylation is a frequent hallmark observed in primary breast tumor (Ordway *et al*. 2007) and its levels are negatively associated with GHSR1a expression (Botla *et al*. 2012).

Despite the obvious association between the presence and changes of ghrelin gene products and breast cancer, limited and conflicting data are available on the functional role that the ghrelin system plays in the regulation of patho-physiologically relevant processes in breast cancer development and progression. Specifically, Cassoni *et al*. showed a decrease in proliferation after ghrelin administration in breast cancer cell lines (Cassoni *et al*. 2004), while other study observed an opposite effect (Jeffery *et al*. 2005). A more consistent explanation seems to be the effect of the In1-ghrelin variant, which, in addition to being overexpressed in breast cancer samples, has been shown to promote basal proliferation in the MDA-MB-231 breast cancer cell line (Gahete *et al*. 2011a).

Prostate Prostate cancer is the most commonly diagnosed malignancy and one of the leading causes of cancer-related deaths in the male population (Siegel *et al*. 2013). Remarkably, prostate cancer development and progression is highly influenced by the endocrine milieu. In this sense, plasma AG, but not UAG or obestatin, has been found to be elevated in prostate cancer patients (Malendowicz *et al*. 2009). In addition, the majority of the ghrelin system components are locally produced in the prostate tissue. Particularly, the expression of ghrelin has been detected in normal prostate, human benign hyperplasia, and prostate carcinoma (Cassoni *et al*. 2004) as well as in prostate cancer cell lines (Jeffery *et al*. 2002, Cassoni *et al*. 2004, Yeh *et al*. 2005). Indeed, locally produced ghrelin seems to be secreted from prostate tissue as it is detected in the medium collected from the PC3 cancer cell line (Jeffery *et al*. 2005). *In vivo*, ghrelin expression seems to be mainly restricted to the glandular epithelial cell cytoplasm with lower staining intensity in the normal glandular tissue compared with malignant prostate epithelium (Yeh *et al*. 2005). Similarly, GOAT expression has also been detected in normal and tumoral prostate tissues and its expression can be modulated by AG, but not by UAG, in normal and tumoral prostate derived cell lines (Seim *et al*. 2013a). In contrast, the presence and abundance of GHSRs (GHSR1a and GHSR1b) in prostate cells are still to be fully defined. Some studies have shown

that GHSR1a is present in a normal prostate cDNA library (Jeffery *et al.* 2002), and absent in carcinomas, while GHSR1b is present in 50% of prostate hyperplasias (Cassoni *et al.* 2004). Likewise, their presence in prostate-derived cell lines is still controversial, in that some studies showed the expression of GHSRs in the majority of cell lines analyzed (ALVA 41, PC3, LNCaP, and DU145) (Jeffery *et al.* 2002), while other studies only reported their presence in a reduced number of prostate cancer cell lines (Cassoni *et al.* 2004). These differences and discrepancies between studies might be explained by the use of different techniques (PCR, ICC, western blot). However, it is clear that further studies are required to unequivocally determine whether GHSRs are present in prostate cells.

Unfortunately, the role of the ghrelin system in prostate cancer cell biology is unclear, probably due to the reduced number of studies performed, and to the conflicting results generated in the reports published to date. In particular, some studies support the idea that AG increases prostate cell line proliferation (Jeffery *et al.* 2005), which could be mediated through the activation of key signaling pathways such as MAPK/ERK and/or PI3K/AKT/mTOR (Yeh *et al.* 2005), and protects against actinomycin D-induced apoptosis (Yeh *et al.* 2005). Conversely, other studies have reported an antiproliferative and proapoptotic effect of AG in prostatic cancer cells (Diaz-Lezama *et al.* 2010, Lawnicka *et al.* 2012). As these studies employed different concentrations of AG, it has been postulated that the physiological levels of AG could act as a positive factor for prostate cells growth, while supra-physiological levels could exert an inhibitory effect. Similarly, the role of ghrelin gene splicing variants is still to be fully elucidated in prostate cells. The Ex3-deleted preproghrelin isoform has been detected in the medium collected from the PC3 cancer cell line; while the In1-ghrelin variant and the recently identified In2c-ghrelin variant (Fig. 1) have also been shown to be expressed in prostate cancer cell lines (Seim *et al.* 2013*b*); however, the pathophysiological role of these variants is still unknown (Jeffery *et al.* 2005, Seim *et al.* 2013*b*). Therefore, further studies are granted to gain insights into the exact role that the ghrelin system exerts in the modulation of prostate tissue biology.

Inflammation/cardiovascular diseases

Ghrelin in inflammation and aging   Several pieces of evidence suggest a central role of the ghrelin system in the control of the inflammatory process and in the evolution of aging. Indeed, plasmatic levels of ghrelin and tissue levels of some of the components of the ghrelin system (ghrelin and its receptors) are regulated during states of acute and chronic inflammation, stress, and/or aging (Baatar *et al.* 2011). In addition, increases in circulating levels of ghrelin have been found in several inflammatory disease states including ulcerative colitis, Crohn's disease, pancreatitis, ankylosing spondylitis, sepsis, colitis, and rheumatoid arthritis (Osawa 2008, Hattori 2009), where its expression levels have also been directly correlated with the severity of the disease and inversely correlated with the expression of proinflammatory cytokines such as IL6, IL1β, and TNFα (Taub 2008). Therefore, these data suggest an important role for the ghrelin system in the regulation of various proinflammatory disease states in humans and in animal models (Dixit & Taub 2005, Mafra *et al.* 2011). Indeed, the use of ghrelin as a therapeutic treatment has been examined in several studies using rodent models of sepsis and endotoxemia, chronic disease, and/or injury (Hattori 2009). Specifically, the use of AG was reported to suppress the expression and/or production of cytokines such as TNFα, IL6, IL1β, and CRP in these models, which was associated with an increase in the survival rate (Taub 2008, Taub *et al.* 2010).

Finally, the idea that the ghrelin system may influence tissue homeostasis and control the local inflammatory state, together with the fact that thymic and splenic expression of the ghrelin system is significantly diminished with advancing age (Taub *et al.* 2010), has led to the conclusion that AG administration to older people could serve as a potential therapy to reduce the increased levels of IL6, IL17, and other inflammatory mediators associated with aging and frailty (Taub *et al.* 2010). However, the role of the ghrelin system in the modulation of the inflammatory process is not fully characterized and cautions should be taken in the interpretation of these results.

Ghrelin in cardiovascular diseases   As mentioned above, the ghrelin system is present in both vascular and cardiac tissues and seems to improve the physiological performance of the cardiovascular system, which has led to the hypothesis that the ghrelin system could exert a protective effect in cardiovascular diseases. Indeed, evidence accumulated during the last decade demonstrated that ghrelin could exert a potential therapeutic benefit in several cardiovascular-related dysfunctions such as myocardial infarction, heart failure, cardiopulmonary bypass, ischemia, atherosclerosis, and/or chronic hypoxia. Specifically, chronic or acute AG treatment in mice has been shown to improve myocardial function and recovery after myocardial infarction, which is often associated with subsequent heart failure (Nagaya & Kangawa 2003).

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In fact, ghrelin has been suggested to have a potential therapeutic benefit in heart failure patients, in whom administration of ghrelin significantly improves left ventricular ejection fraction, increases peak workload and peak oxygen consumption during exercise (Kishimoto et al. 2012), improves cardiac function, and decreases systemic vascular resistance (Nagaya & Kangawa 2003). Similarly, AG treatment reduces inflammatory response, apoptosis and oxidative stress, and preserves the cardiac pumping function in a rat model of cardiopulmonary bypass through GHSR1a and Akt signaling (Cao et al. 2013). In addition, AG exerts a protective effect against ischemia/reperfusion injury (Chang et al. 2004), probably through a direct effect on the heart (Kishimoto et al. 2012) and also exerts a vasodilatory effect in the vasculature (Isgaard & Granata 2011, Lacerda-Miranda et al. 2012), which may be of potential importance for the development of atherosclerosis. Finally, a therapeutic benefit of ghrelin in the treatment of pulmonary hypertension has also been suggested, particularly in subjects prone to chronic hypoxia, a state that elevates pulmonary arterial pressure and increases the wall thickness of peripheral pulmonary arteries, because ghrelin treatment can attenuate, in rats, the hypoxia-induced development of pulmonary arterial hypertension and pulmonary vascular remodeling (Kishimoto et al. 2012).

The pathophysiological implications of the ghrelin system in cardiovascular diseases is further supported by epidemiological studies that indicate that single nucleotide polymorphisms (SNPs) in the ghrelin gene, which influence ghrelin levels in the circulation, could be associated with an altered hypertension risk (Liu et al. 2011). In addition, SNPs in the GHSR gene could also be markers for cardiac diseases, in that allele frequencies in the regulatory region of GHSR differ significantly between individuals with or without left ventricular hypertrophy (Liu et al. 2011). Unfortunately, to the best of our knowledge, the role of other components of the ghrelin system in cardiovascular diseases has not been explored to date.

Neurodegeneration

Recent data demonstrating that ghrelin is involved in neuroprotection, together with the wide distribution of GHSRs in many brain areas, have reinforced the idea that changes in the ghrelin system could be implicated in the development and/or progression of some neurodegenerative diseases such as AD and PD.

Alzheimer's disease AD is the most prevalent age-related neurodegenerative disease and is markedly influenced by metabolic and inflammatory status. AD is characterized by a marked impairment in memory and learning processes, two cognitive functions in which the ghrelin system has been shown to play a relevant role. Indeed, in 2002, it was reported that plasma ghrelin levels were lower in older subjects than in young subjects, providing the first evidence for an age-related decline in plasma ghrelin concentration (Rigamonti et al. 2002). Although later studies showed that ghrelin levels did not vary in the cerebrospinal fluid of AD patients when compared with age-matched controls (Proto et al. 2006), more recent studies provide strong evidence to suggest an association between dysregulations in the ghrelin system and AD incidence. Particularly, our group analyzed the mRNA expression levels of the ghrelin system in three different regions of the temporal gyrus of control and AD human brains, showing for the first time that AD patients had a reduction in local brain ghrelin production compared with age-matched controls (Gahete et al. 2010b). Moreover, In1-ghrelin and the GOAT enzyme were also present in the temporal lobe and were down-regulated in AD (Gahete et al. 2010b). This study also revealed that the ratio between GHSR1a and GHSR1b receptors was altered in AD patients, suggesting that these changes in the ghrelin system could be involved in the severity of the disease. The fact that recent data indicate that SNPs of the ghrelin gene are associated with the age of AD onset (Shibata et al. 2011), together with the observation that male patients with AD have a disruption in the normal compensatory modulation of ghrelin secretion (Theodoropoulou et al. 2012), strongly reinforce the idea that the ghrelin system should be considered as a potential therapeutic target in the treatment of AD-related comorbidities.

Parkinson's disease PD is the second most common neurodegenerative disorder. The disease affects the motor system and results from the progressive degeneration of dopamine neurons in the pars compacta of the substantia nigra (SNpc; Bisaglia et al. 2013). The limitations of the current dopamine-replacement therapy have led to extensive investigation of novel nondopaminergic drugs that may provide treatment for PD symptoms (Bisaglia et al. 2013). Despite the limitations in the generation of animal and cellular models of PD that accurately resemble the processes known to occur during PD-associated cell death, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated animal models have emerged as a widely used approach for studying PD pathobiology (Langston & Ballard 1983). Due to the positive role of the ghrelin

system in the promotion of neural survival and in the dopaminergic-system function, an increasing number of studies have investigated, over the last years, the effects of ghrelin in different animal models. These studies have suggested that ghrelin might exert an effect in the treatment of PD. Specifically, it has been demonstrated that ghrelin can inhibit the MPTP-induced dopaminergic neuronal loss and dopamine depletion in mice (Jiang *et al.* 2008), probably through a dual neuronal-microglial action, in that ghrelin can protect the normal dopamine function by activating UCP2-dependent mitochondrial mechanisms (Andrews *et al.* 2009) and can act as a survival factor for dopaminergic neurons by functioning as a microglia-deactivating factor (Moon *et al.* 2009). The fact that GHSRs are expressed in the SNpc (Andrews *et al.* 2009) has reinforced the potential value of the ghrelin system as a novel, palliative, and neuroprotective target in neuro-degenerative diseases such as PD. However, further studies are required in order to fully elucidate the implications and putative therapeutic use of the ghrelin system in PD-associated neuronal impairment.

Concluding remarks and future directions

Over 14 years have passed since the discovery of ghrelin (Kojima *et al.* 1999). Since that time, an emerging body of evidence has grown to support the notion that several components of the ghrelin system are expressed in a wide variety of tissues, where they play crucial roles by regulating multiple biological functions. However, this evidence also revealed that the ghrelin system is far more complex than it was originally envisioned. The complexity of this system lies on the existence of multiple ghrelin gene-derived variants, receptors, and interrelated enzymes (e.g., GOAT), which have been found to be expressed throughout the body, and also on the fact that the ghrelin system may play a crucial role in the regulation of a variety of pathophysiological processes (e.g., development and pro-gression of endocrine-related tumors, inflammatory/cardiovascular diseases and neurodegeneration). The data summarized in this review highlights the conception that ghrelin is one of the components of a complex, intricate regulatory system composed of a growing number of alternative peptides, regulatory enzymes, known and unknown receptors, which can interact with them as well as with other regulatory systems in order to tightly modulate relevant biological and pathophysiological processes. However, several relevant questions regarding the composition and function of the ghrelin system in different tissues and cells types still remain unknown.

Therefore, additional efforts from the scientific community will be necessary to better understand the composition, functions, and relationships among the different com-ponents of the ghrelin system under normal (biological/physiological) conditions, as well as in pathological states, by continuing to employ, refine, and develop new and more representative *in vitro* and *in vivo* experimental approaches targeting the ghrelin system.

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