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Functional hypothalamic amenorrhea seen from different perspectives

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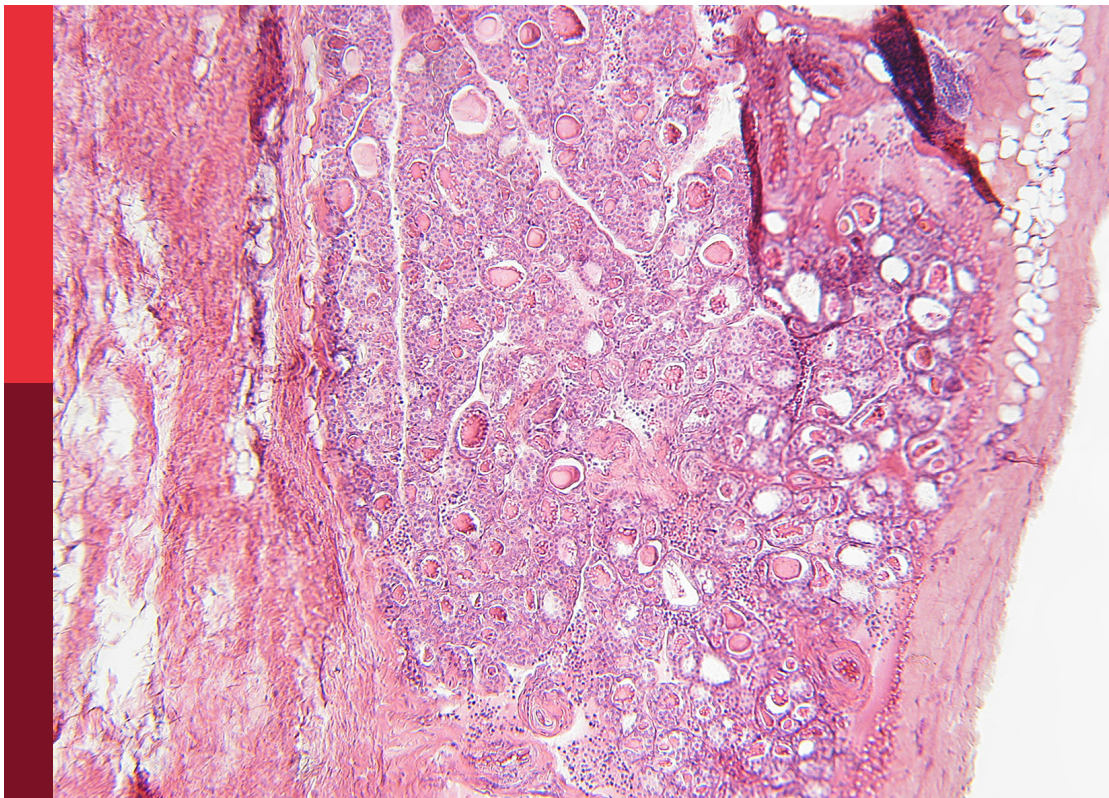

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Functional hypothalamic amenorrhea seen from different perspectives

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Table of contents

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Editorial: Functional hypothalamic amenorrhea seen from different perspectives

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KEYWORDS

amenorrhea, hypogonadotropic hypogonadism, osteoporosis, polycystic ovarian morphology, in vitro fertilization (IVF)

Editorial on the Research Topic:
Functional hypothalamic amenorrhea seen from different perspectives

Functional hypothalamic amenorrhea (FHA) often represents a diagnostic and therapeutic challenge and affects the most diverse areas of medical clinics; to better understand its multifaceted characteristics, we tried to approach this disorder from different perspectives.

FHA is a form of Hypogonadotropic Hypogonadism (HH) in which anovulation and estrogen deficiency are expressed by inadequate endometrial growth, absence of menstruation, and infertility and can have lasting adverse effects on bone mineral density and trophism of genital and other target tissues (1). It can be related to psychological stress, decreased energy intake and strenuous exercise (2). FHA is considered "functional" because it can regress through correction or improvement of the behavioral factors that cause it.

Stress conditions play a primary role in promoting FHA. In the presence of similar stressors, interindividual variability in stress response results in inhibition of the HPG axis in some women and not in others. Recent studies, comprehensively reviewed by Fontana et al., have demonstrated a genetic contribution to FHA. Rare or polymorphic variants in genes controlling the development and/or function of GnRH neurons have been recognized in both idiopathic HH and FHA women, suggesting the presence of inherited susceptibility to functional impairment of GnRH secretion. Epigenetic changes have also been associated with different pathways involved in the HPG axis and, therefore, may participate in FHA and confer a personal predisposition to anovulation. Federici et al., in a large cohort of patients with congenital HH (CHH), identified gender differences in clinical presentation that could indicate variable expression of rare genetic variants.

The effect that long-term exposure to stress has on energy metabolism and reproduction is almost certainly caused by alterations in kisspeptin secretion from the arcuate nucleus of the hypothalamus, resulting in reduced GnRH drive. Although this effect

was thought to be mediated by glucocorticoid receptors on kisspeptin neurons (3), Huang et al., using a special mouse model, have elegantly demonstrated the secondary role of these receptors and the possible involvement of receptors, including those for insulin, adiponectin, or leptin.

The diagnosis of FHA is based on clinical presentation and plasma hormone assays, both of which are characteristic (1). However, because some FHA patients with polycystic ovary morphology share features with a certain phenotype of women with polycystic ovary syndrome (PCOS), the differential diagnosis between these two disorders may not be easy. In fact, these are the two most frequent causes of secondary amenorrhea. Over the past two decades, some authors have proposed that these conditions may coexist (4, 5), but the majority support the clear distinction between them. Beitlt al. demonstrated that plasma hormone assays can be adequately sensitive to distinguish FHA women from PCOS women and proposed an original linear discriminant model using plasma assays of testosterone, SHBG, and gonadotropin.

One of the most important and potentially lasting consequences of FHA is bone loss secondary to metabolic and endocrine imbalances. Indirli et al. provided a thorough review on this issue, emphasizing the need for comprehensive clinical and instrumental evaluation, the utility of lifestyle interventions, and an optimal estrogen replacement strategy. Behary et al. reviewed the available evidence on several alternative and novel pharmacological interventions for the treatment of FHA-related bone loss, in addition to transdermal 17β-estradiol, which is currently the preferred intervention.

The low nutrient availability due to reduced dietary intake and/or high-energy exercise in women with FHA deserves to be rebalanced. The negative effects of hypoestrogenism on target tissues can be partially offset by hormone replacement treatment (HRT), which is more effective on uterine trophism than on skeletal homeostasis. For patients with fertility needs, ovarian stimulation with gonadotropins or pulsatile GnRH is used to induce ovulation. Pulsatile GnRH would be the most physiological-like method, but it requires close monitoring and its compliance is made difficult by the use of a portable pump injection device. In clinical practice, the most used ovulation induction procedure is daily gonadotropin injection, usually followed by oocyte retrieval, *in vitro* fertilization (IVF), and embryo transfer. Retrospectively evaluating the reproductive outcome of 81 patients with HH and 112 controls who underwent an IVF procedure, Zhang et al. showed that there were no significant differences between the two groups in IVF-related parameters and cycle outcomes. Since FHA patients are characterized by very low gonadotropin levels, it was hypothesized that in addition to stimulation with FSH, serum LH supplementation may be necessary to promote meiosis and the final stages of antral follicular growth (6). Di Segni et al. set out to evaluate the possible effect of exogenous LH supplementation on fertility-related outcomes in women with FHA. They concluded that available data demonstrate a positive effect on IVF cycle outcomes and support LH supplementation in women with FHA.

Although a psychogenic component has been recognized in FHA since its first diagnostic formulation (7), the role of psychological factors in the onset or persistence of this disorder is still poorly understood. Bonazza et al., analyzing published evidence, identified some recurrent psychological factors that could be clinically relevant, such as depression and dysfunctional attitudes toward eating, a higher level of perfectionism, greater concerns about errors, and a greater need for approval than controls. A tailored behavioral psychological intervention offers an effective treatment option that complements the clinical approach aimed at the recovery of ovarian activity.

Since heart rate variability (HRV) is a reliable measure of psychophysiological response to stress and coping to stimuli, Maiorana et al. examined changes in HRV during observation of erotic, neutral and disgusting images in a group of patients with FHA compared with controls. The results showed that patients with FHA had significantly higher HRV activation. This elevated HRV reactivity could reflect maladaptive activation of the parasympathetic nervous system and reduced reactivity with a delayed response to meet the environment demands.

A multidisciplinary approach is critical in the clinical management of FHA; it must involve a collaborative process among gynecologists, geneticists, endocrinologists, neurologists, clinical psychologists, and psychiatrists to facilitate proper diagnosis and provide the most appropriate treatment.

Author contributions

EG and AM conceived the project; AL, AP, MM and EV created the prerequisites and encouraged the collection of contributions. All authors collaborated in the successful development of the Research Topic. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Hypothalamic Kisspeptin Neurons Regulates Energy Metabolism and Reproduction Under Chronic Stress

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Background: Stress activates the hypothalamic-pituitary-adrenal (HPA) axis, affecting energy homeostasis and reproduction. The aim of this study was to investigate whether stress affected energy metabolism and reproduction through the glucocorticoid receptor on Kisspeptin neurons in the hypothalamus.

Methods: Four groups included control group, chronic restraint stress group, Kisspeptin specific glucocorticoid receptor knock out group (KGRKO) and KGRKO+stress group. Body weight, food intake, estrous cycle of female mice, serum sex hormone levels, serum corticosterone and prolactin, Kisspeptin expression in the hypothalamus were measured.

Results: The restraint stress group showed a significant weight loss compared with the control group. KGRKO+restraint stress group had a reduced weight loss, suggesting that restraint stress might partially affect the energy metabolism through GR on Kisspeptin neurons. In terms of reproductive function, the restraint stress group and the KGRKO+ restraint stress group showed missing pre-estrus period or prolonged estrous cycles. Serum LH and FSH in KGRKO + restraint stress group decreased significantly compared with KGRKO group. However, no significant difference in the level of serum testosterone was observed. After restraint stress, the levels of serum cortisol and prolactin in male and female mice were significantly higher than the control group, and the hypothalamus Kiss1 gene mRNA expression and Kisspeptin protein expression were significantly decreased.

Conclusion: Chronic restraint stress induced weight loss and negative changes in reproduction, which were partially mediated by glucocorticoid receptor on Kisspeptin neurons in the hypothalamus.

Keywords: reproductive function, energy metabolism, glucocorticoid, restraint stress, Kisspeptin

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INTRODUCTION

With the rapid development of modern society, people are under great pressures. Stress may lead to physiological responses including decreased appetite, increased blood pressure and heart rate, reproductive dysfunction, insomnia, and even anxiety and depression, which brings huge economic burdens and losses to society.

As a stress response, the hypothalamic-pituitary-adrenal axis (HPA) is activated and glucocorticoids (glucocorticoid, GC) are secreted through the adrenal glands (1). long-term improper stress can affect normal physiological functions including energy metabolism and reproductive function. Studies have found that long-term exposure to stress reduces the body weight and food intake of rodents. Rats exposed to acute or chronic restraint stress have significant changes in blood lipids and lipoprotein levels, and plasma free fatty acids and cholesterol levels increase. And the triglyceride level is reduced. Disorders of reproductive function have also been observed in patients with anorexia nervosa (2) and girls with severe psychological trauma (3). The common feature of these people is that they have been activated on the HPA axis for a long time, and their cortisol levels are higher than normal. In population studies, it was also found that the blood cortisol level of prepubertal girls was positively correlated with the age of menarche (2). Famine exposure is also a kind of stress response. Our research team analyzed the relationship between famine exposure and reproductive function in 2868 women in a previous study. We found that exposure to famine during childhood increases the incidence of premature menopause and even premature ovarian failure (4). In animal experiments, it can also be observed that the mother is exposed to glucocorticoids during pregnancy and the estrus cycle is delayed after the offspring is born (5). Therefore, stress will affect the body's energy metabolism and reproductive function.

What are the specific mechanisms by which stress affects energy metabolism and reproductive function? Studies showed that central mechanism, especially the hypothalamus, might be involved (6).

Kisspeptin neurons are mainly distributed in the hypothalamic arcuate nucleus (ARC), and the third paraventricular anterior ventral nucleus (AVPV) (7). It can directly act on hypothalamic GnRH neurons to promote the secretion of GnRH, resulting in hypothalamic-pituitary-gonadal (HPG) Activate, causing reproductive function to be affected. Kisspeptin plays an important role in reproductive function (8). One of the reasons for hypogonadotropic sexual dysfunction is the mutation of Kiss1 gene. Therefore, Kisspeptin is the key to start and maintain the function of the HPG axis (9). In addition, there are insulin and adiponectin receptors on Kisspeptin neurons, which can sense metabolic changes and regulate energy (10). Therefore, Kisspeptin may be a bridge between energy metabolism and reproductive function.

Glucocorticoid receptor (GR) is expressed on Kisspeptin neurons in the mouse hypothalamus (11), which suggests that the regulation of glucocorticoids on reproductive function may be through the Kisspeptin neurons in the hypothalamus. Kinsey-Jones et al. (12) observed that the expression of kisspeptin mRNA in the hypothalamus of female rats decreased under different stress conditions, and the results of our group's previous studies also confirmed that dexamethasone can inhibit hypothalamic GT1-7 neuronal cells (GnRH, Kisspeptin Both expression and secretion) The transcriptional expression of Kiss1 gene mRNA and the expression level of Kisspeptin protein, and the glucocorticoid receptor blocker RU486 can antagonize this effect. These findings suggest that hypothalamic Kisspeptin neurons may be stress-affected energy A new important central target of metabolism and reproductive function.

In this study, we established a mouse model of chronic restraint stress to observe the influence of chronic restraint stress (CRS) on energy metabolism and reproductive function, as well as the influence of kisspeptin expression in the hypothalamus. We further constructed the Kisspeptin neuron-specific glucocorticoid receptor knockout mice (Kisspeptin specific glucocorticoid receptor knock out, KGRKO) undergo CRS which helps to reveal the role of stress in regulating energy metabolism and reproductive function through the central nervous system, to further elucidate the interaction mechanism between HPA and HPG axis and the relationship between stress-induced hypercortisolemia and reproductive diseases.

MATERIALS AND METHODS

Laboratory Animals and Reagents

Kisspeptin specific glucocorticoid receptor knock out (KGRKO) mice was accomplished by crossing mice engineered with lox P sites flanking exons 1C and 2 of the mouse GR gene (GRloxP) with mice expressing Cre recombinase driven by the Kiss1 Cre promoter (Kiss1Cre) on a C57BL/6J background. Male and female KGRKO mice (homozygous for GR flox [GR flox/flox] and expressing Kiss1Cre), here denoted as GRflox/floxKIss1cre, and littermate control mice (GRflox/floxKiss1cre) containing only the GR flox allele (no Kiss1-Cre transgene) were used for all studies and were 8–10 weeks old at the beginning of each experiment. To generate litters expressing both of these genotypes, the female breeders were GRflox/floxKiss1cre-, whereas the male breeders were GRflox/floxKiss1cre+.

All mice were housed under standard conditions (constant temperature, constant humidity conditions, and a 12-h light/dark cycle), 5 mice a cage, with free access to food and water. The study followed the National Guidelines for Laboratory Animal Welfare and was approved by the Experimental Animal Ethics Committee of Fujian Medical University.

Establishment of Mice Model

There were four groups, that were control group, chronic restraint stress group (stress group), Kisspeptin specific glucocorticoid receptor knock out group (KGRKO group) and KGRKO+stress group, 10 mice for each group. Twenty mice of wildtype mice and KGRKO mice were randomly divided into control and stress group, respectively.

Stress mice were placed in a 50ml centrifuge tube. The top and side walls of the centrifuge tube have small holes with a diameter of 0.5 cm to ensure air flow. The restraint stress is performed for 1 hour from 9:00 to 10:00 in the morning. During the restraint period, the animal has no food or water. After 1 hour restraint, the mice were immediately returned to their cages, and they were free to eat and drink. The unrestrained stressed mice (control group) were kept in the cage.

Investigators could not be blinded to the mouse strain due to that the stress group mice were placed in a tube.

Serum Measurement

Serum sex hormone levels, serum corticosterone and prolactin were measured with ELISA Kit, following the manufactory instructions.

Expression of Kisspeptin in the Hypothalamus by Immunofluorescence

The animals were perfused with phosphate buffered solution (PBS) at a pH of 7.4 by a cannula into the left ventricle after anaesthesia, followed by 4% paraformaldehyde. After perfusion, the brains were immediately removed and were fixed in 4% paraformaldehyde in PBS at 4°C for 12 h and passed through 20 and 30% sucrose gradients prior to embedding in optimum cutting temperature compound (OCT). 20μm tissue sections were air-dried at −20°C and moved to −80°C for long-term storage. Immunofluorescence was performed according to the manufacturer's instructions for fixed-frozen tissue. Glass coverslips were fixed with 4% paraformaldehyde, 0.1% triton X-100 permeabilized, blocked with 1% BSA, and incubated with the Kisspeptin antibody (Mouse monoclonal antibody; Abcam, USA; 1: 500) at 4°C overnight. After washes with 1×PBS, cells were incubated with the corresponding secondary antibody conjugated with Alexa Fluor® 647 (Donkey polyclonal Secondary Antibody to mice IgG - H&L; Abcam, USA; 1:100) in dark for 1 h and analyzed using Inverted fluorescence microscope (Leica Microsystems Ltd. CH-9435 Heerbrugg. Type: DFC425 C(12730222). Serial No: 533481211. Leica, Germany). 4′, 6-diamidino-2-phenylindole (DAPI) was used to label nucleus. ImageJ was used to calculate the expression of Kisspeptin fluorescence intensity per scaffold. At least 3 slides were examined in each treatment group for each experiment. A comparison of the fluorescence intensity of Kisspeptin between the indicated groups was performed.

Quantitative RT-PCR (qRT-PCR)

At the end of each experiment, a microdissection procedure was used to isolate hippocampus. Total RNA was extracted with TRIzol (RNAiso Plus)method (Takara, Japan). RNA was reversed transcribed into cDNA using the two-step method with PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan), according to the manufacturer's instructions. mRNA qRT-PCR was performed with the TB Green™ Premix Ex Taq™ (TliRNaseH Plus) (Takara, Japan) according to the manufacturer's instruction. The procedure was 95°C for 1 min; 95°C for 15 s and 60°C for 34 s, for 40 cycles; 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The primers were shown in **Table 1**.

Statistical Analyses

All statistical analyses were performed using the SPSS Statistics 20 software. Data have been expressed in terms of mean ± standard deviation. Statistical significances between two groups of data were determined using unpaired, two-tailed Student's *t*-test. A P value >0.05 was not considered significant, P value <0.05 was labeled as (*), P value <0.01 was labeled as (**), P value <0.001 was labeled as (***).

TABLE 1 | Primers of qRT-PCR.

Gene		Primers sequence
β-actin	Forward	5' CTACCTCATGAAGATCCTGACC 3'
	Reverse	5' CACAGCTTCTCTTTGATGTCAC 3'
$GR^{flox/flox}$	Forward	5'-ATGCCTGCTAGGCAAATGAT-3'
	Reverse	5'-TTCCAGGGCTATAGGAAGCA-3'
Kisspeptin	Forward	AGCTGCTGCTTCTCCTCTGT-3'
	Reverse	AGGCTTGCTCTCTGCATACC-3'

β-actin was used as mRNA reference gene, with the 2−ΔΔCt method used for quantitation. Triplicate experiments were performed and repeated at least 3 times.

RESULTS

The Effect of Chronic Restraint Stress on the Body Weight of KGRKO Mice

In order to understand the effect of GR signals on Kisspeptin neurons on the body weight of mice under chronic restraint stress, we performed chronic restraint stress on mice for 28 days and monitored their body weight every day.

The mice were divided into four groups: control group, restraint stress group, KGRKO group, KGRKO+ restraint stress group, and weight and food intake were measured every day for 28 days. As shown in **Figures 1A, B**, after 28 days of intervention, the restraint stress group compared with the control group, and the KGRKO+ restraint stress group compared with the KGRKO group, the body weight was significantly reduced. Compared with the restraint stress group, the weight loss of the KGRKO+ restraint stress group was reduced.

As shown in **Figures 1C, D**, after 28 days of intervention, the restraint stress group compared with the control group, and the KGRKO+ restraint stress group compared with the KGRKO group, the body weight was significantly reduced. Compared with the restraint stress group, the weight loss of the KGRKO+ restraint stress group was reduced, suggesting that the influence of restraint stress on energy metabolism may partly act through the GR on the Kisspeptin neurons.

The Effect of Chronic Restraint Stress on the Estrous Cycle

In order to understand the influence of GR signals on Kisspeptin neurons on the estrous cycle of mice under chronic restraint stress, we performed vaginal smears every day to observe the estrous cycle 10 days before restraint stress.

From 10 days before restraint stress to 28 days before restraint stress ended, lasting 38 days, vaginal smears were performed every day. As shown in **Figure 2**, the control group showed a regular estrus cycle of 4–5 days, while the restraint stress group and KGRKO+ restraint stress group showed a lack of proestrus or a prolonged estrus cycle.

The Effect of Chronic Restraint Stress on Serum Sex Hormone

In order to understand the influence of GR signals on Kisspeptin neurons on the levels of sex hormones in mice under chronic restraint stress, we collected blood from the orbit after the experiment, and separated serum to detect LH, FSH and

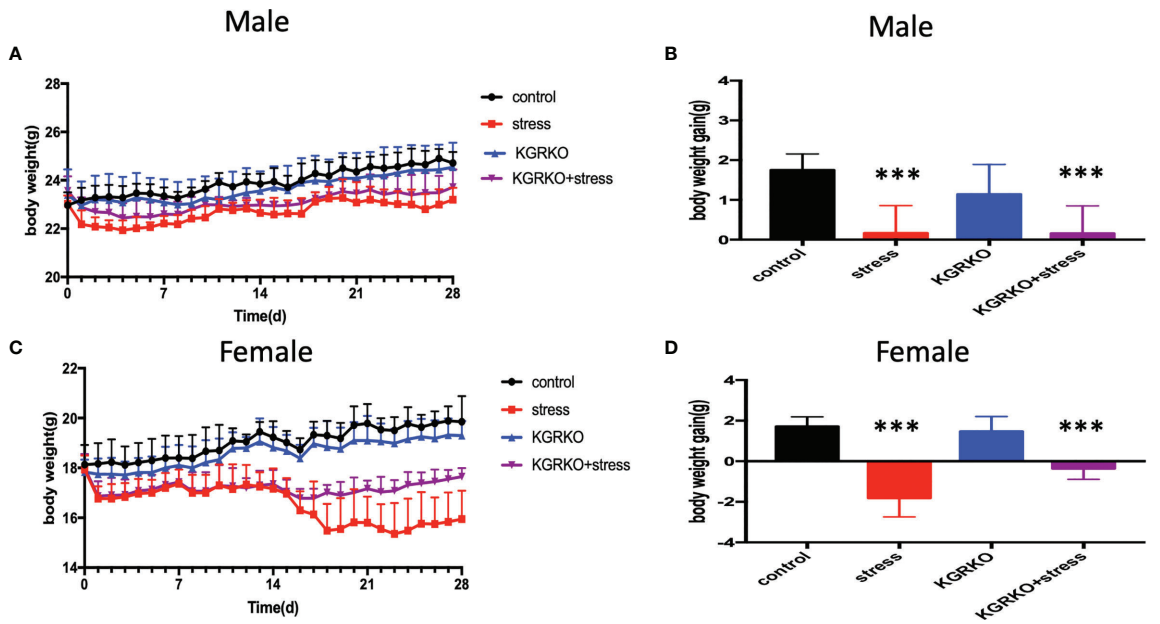

FIGURE 1 | The effect of chronic restraint stress on the body weight of KGRKO mice. control: control group, stress: restraint stress group. KGRKO group: Kisspeptin neuron-specific GR knockout mice. KGRKO+stress group: Kisspeptin neuron-specific GR knockout mice restraint stress group. **(A, B)** The effect of chronic restraint stress on the body weight of male KGRKO mice. **(C, D)** The effect of chronic restraint stress on the body weight of male KGRKO mice. ***Represents the comparison with the control group, P<0.001.

estrogen levels after centrifugation. As shown in **Figures 3A–C**, compared with the control group, the female mice restraint stress group and the KGRKO group showed a significant decrease in serum LH and FSH, and the difference was statistically significant, while the serum estrogen level had no significant difference (P> 0.05). Compared with the KGRKO group, the serum LH and FSH of the KGRKO+ restraint stress group decreased, and the difference was statistically significant (P<0.05), but there was no

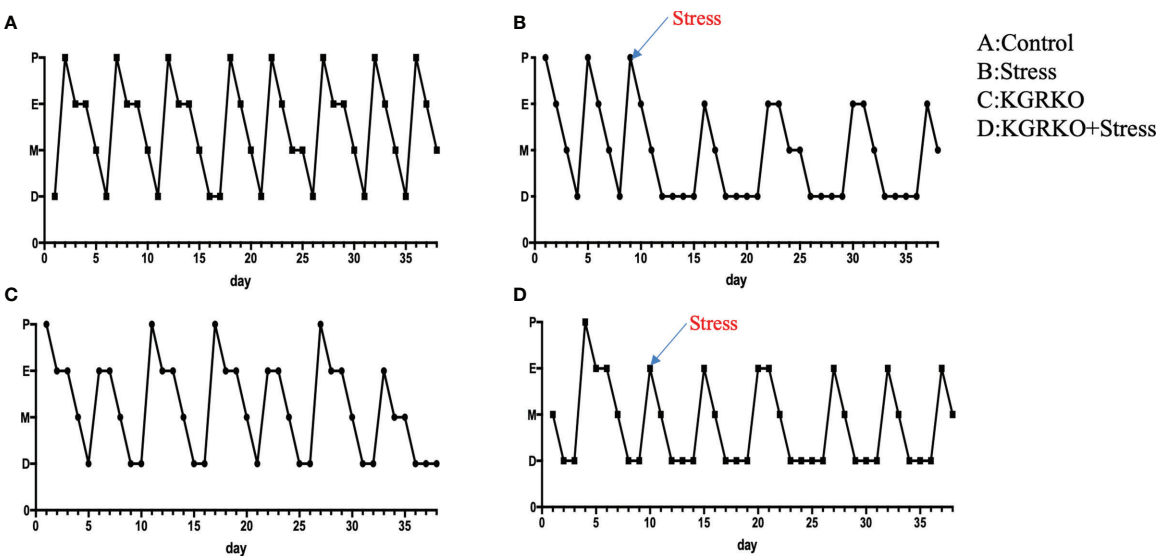

FIGURE 2 | The effect of chronic restraint stress on the estrous cycle of female KGRKO mice. Estrous cycle included 4 states, P, Proestrus; E, Estrus; M, Metestrus; D, Diestrus. **(A)** control group, **(B)** restraint stress group. **(C)** KGRKO group, Kisspeptin neuron-specific GR knockout mice group. **(D)** KGRKO+ restraint stress group, Kisspeptin neuron-specific GR knockout mice restraint stress group.

significant difference in the serum estrogen level (P>0.05). As shown in **Figure 3D**, there was no significant difference in serum testosterone levels between male restraint stress group, KGRKO group and KGRKO+ restraint stress group (P>0.05).

Effect of Chronic Restraint Stress on Serum Cortisol Level in Mice

In order to explore the effects of chronic restraint stress on cortisol and prolactin in mice, after 28 days of restraint stress, the mice were subjected to orbital blood collection, and the serum was separated to detect the levels of cortisol and prolactin after centrifugation, as shown in **Figures 4A, B**. It can be seen that the serum cortisol in the restraint stress group was significantly higher than that in the control group (P<0.001). As shown in **Figures 4C, D**, we can see that whether it is male or female, the serum prolactin of the restraint stress group was significantly higher than that of the control group (P<0.001).

Effect of Chronic Restraint Stress on the Expression of Kisspeptin Protein in Mouse Hypothalamus

In order to explore whether chronic restraint stress affects the expression of KiSS1 gene mRNA in hypothalamus, at the end of the experiment, we isolated mouse hypothalamus, extracted hypothalamic mRNA, and detected the expression of KiSS1 gene mRNA in hypothalamus by Real-time PCR. The results showed that compared with the control group, whether it was male or female mice, after 28 days of restraint stress, the expression of KiSS1 gene mRNA in the hypothalamus decreased significantly, as shown in **Figure 5**. And the expression of Kisspeptin protein in hypothalamus was detected by immunofluorescence method. As shown in **Figure 6**, the expression of Kisspeptin in the hypothalamus of the chronic restraint stress group was lower than that of the control group, which is consistent with the effect of chronic restraint stress on the transcription and expression of hypothalamic Kiss1mRNA.

DISCUSSION

The main findings in our present study include (1) Chronic restraint stress induced weight loss in mice and reversed body weight gain induced by high-fat diet. Chronic restraint stress played a negative role in regulating reproductive function. (2) the KGRKO+restraint stress group had a reduced weight loss compared with the restraint stress group,; (3) the restraint stress group and the KGRKO+restraint stress group showed missing pre-estrus period or prolonged estrous cycles. (4) Serum LH and FSH in KGRKO + restraint stress group decreased significantly compared with KGRKO group.

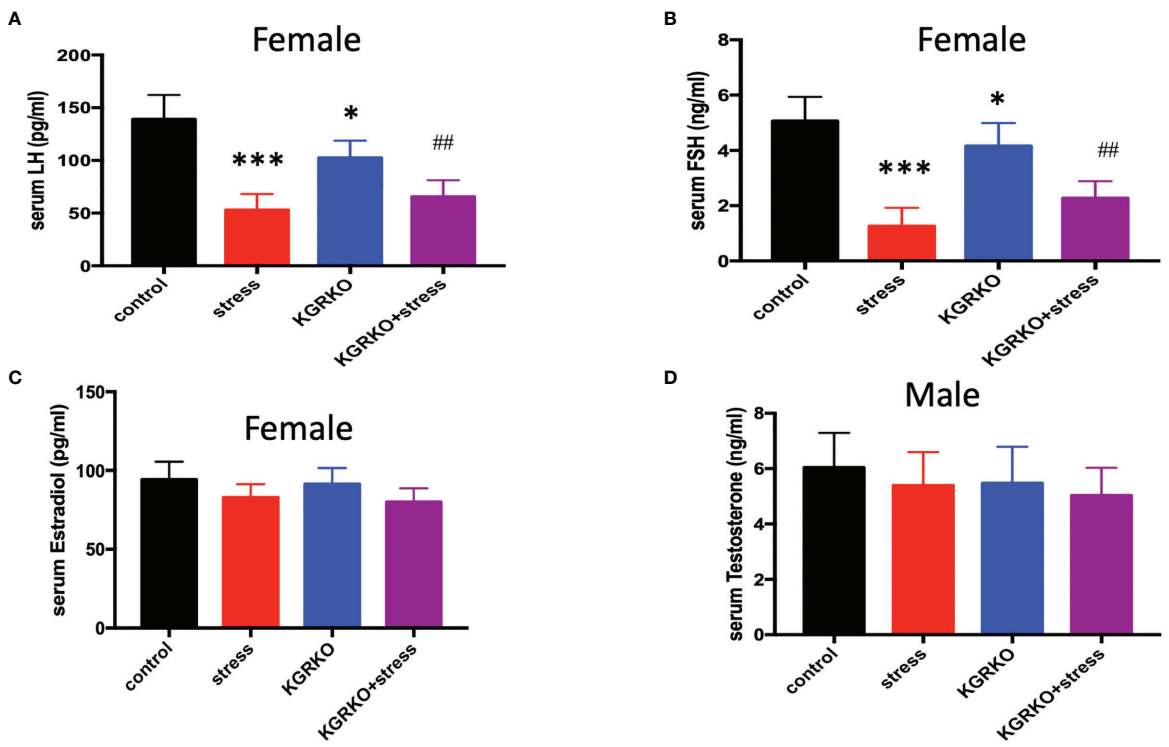

FIGURE 3 | The effect of chronic restraint stress on sex hormones in KGRKO mice. **(A)** LH level in female mice; **(B)** FSH level in female mice; **(C)** Estrodiol level in female mice; **(D)** Testosterone level in male mice; control: control group, stress: restraint stress group. KGRKO group: Kisspeptin neuron-specific GR knockout mice. KGRKO+stress group: Kisspeptin neuron-specific GR knockout mice restraint stress group. *Represents the comparison with the control group, P<0.05. ##Represents the comparison with the KGRKO group, P < 0.01. ***Represents the comparison with the control group, P < 0.001.

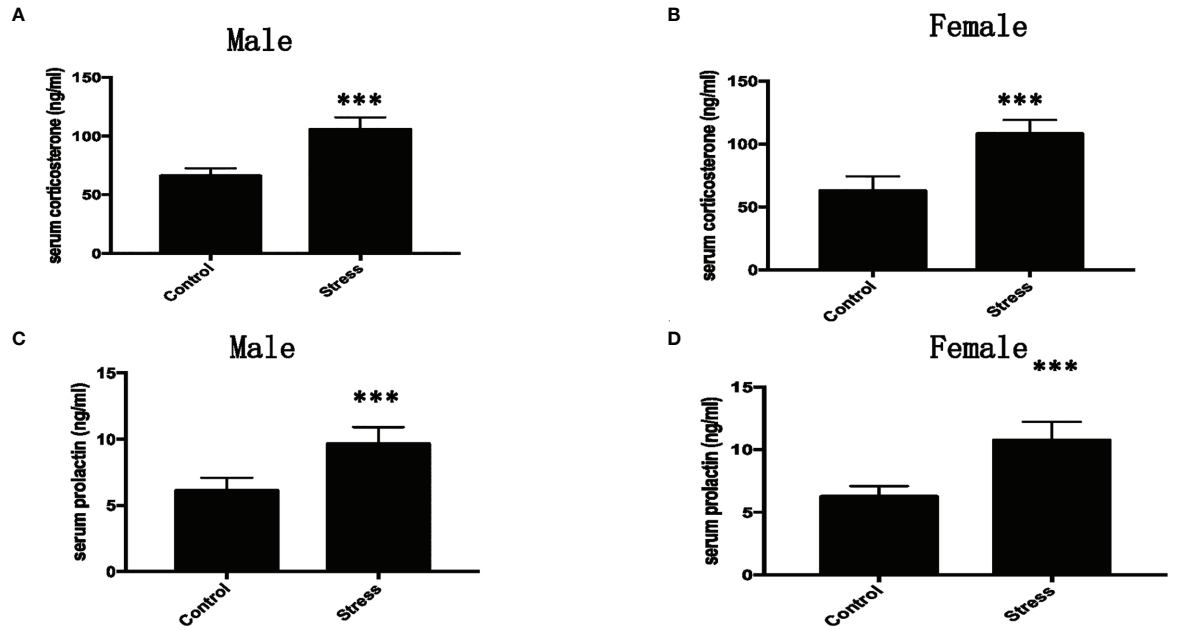

FIGURE 4 | The effect of chronic restraint stress on serum cortisol and prolactin. **(A)** Cortisol in male mice; **(B)** Cortisol in female mice; **(C)** Prolactin in male mice; **(D)** Prolactin in female mice. control: control group, stress: restraint stress group. n=10. ***Represents the comparison with the control group, P<0.001.

Stress activates the hypothalamic-pituitary-adrenal axis, promotes the synthesis and release of glucocorticoids, thereby affecting the expression and regulation of target tissue genes. Mice under chronic stress showed elevated basal cortisol levels (13, 14), and this result may reflect the changes in the body's sensitivity to the negative feedback effects of circulating glucocorticoids (15). In this study, we found that the levels of cortisol and prolactin in restraint stress mice increased, which is consistent with the physiological response that repeated stress caused the HPA axis to be activated (16–18).

The HPA axis is involved in the regulation of energy metabolism. Under stress, the synthesis and release of adrenal glucocorticoids increase. Food intake and many metabolic processes are mediated by glucocorticoids. Therefore, adrenal glucocorticoids mediate changes in the body's energy and metabolic requirements (19). For example, glucocorticoids can promote liver gluconeogenesis to ensure energy supply. In addition to stress affecting energy metabolism (20), it also has an inhibitory effect on the hypothalamic-pituitary-gonad (HPG) axis. We already know that glucocorticoids can act on the center and play a negative feedback effect on the HPA axis. So what is the

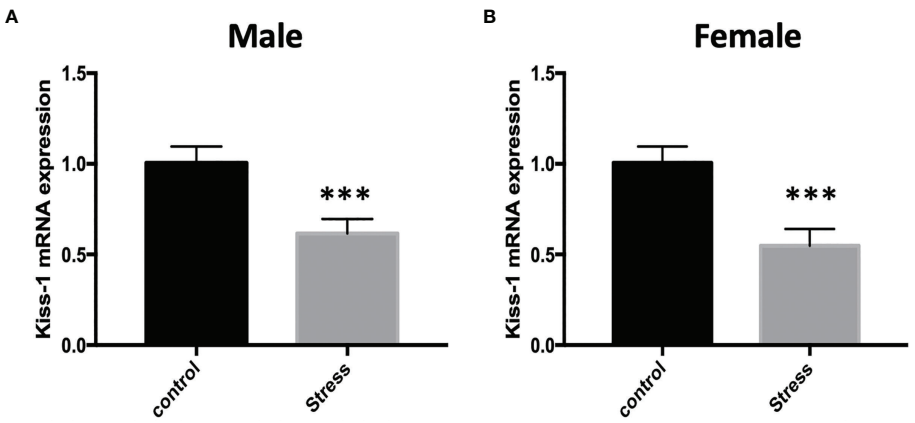

FIGURE 5 | The effect of chronic restraint stress on the expression of Kiss1 mRNA in the hypothalamus. control: control group, stress: restraint stress group. n=5. ***Represents the comparison with the control group, P<0.001. **(A)** Kiss-1 mRNA expression after chronic restraint stress in male mice; **(B)** Kiss-1 mRNA expression after chronic restraint stress in female mice.

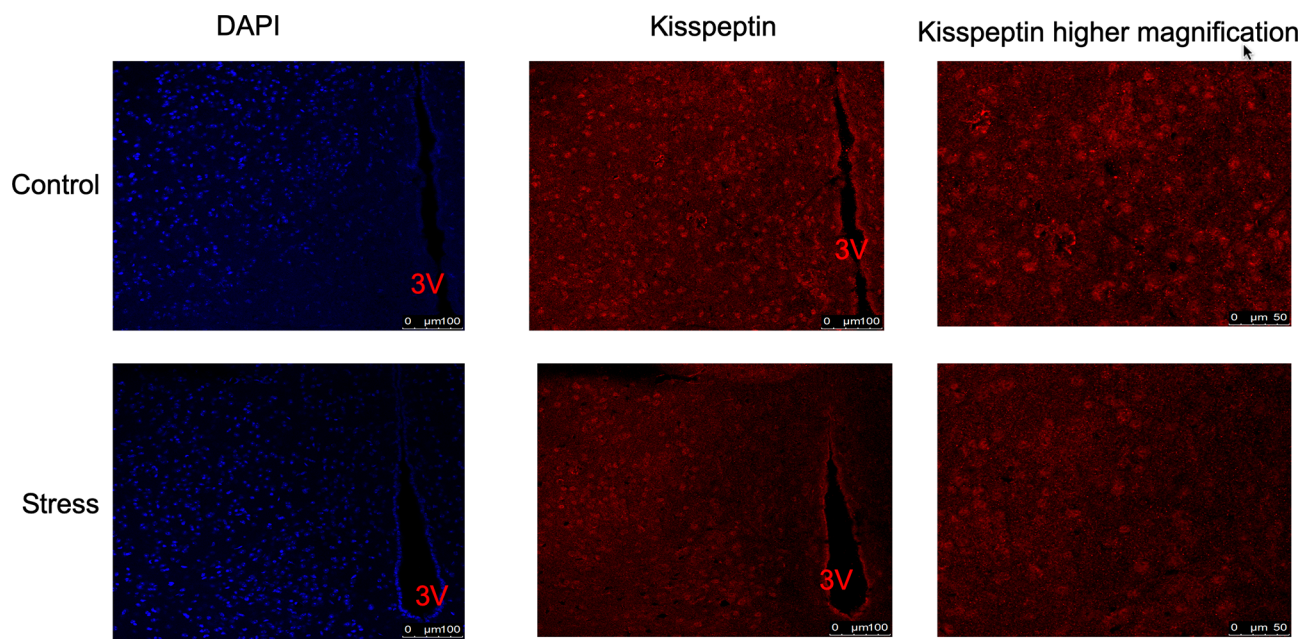

FIGURE 6 | The effect of chronic restraint stress on the expression of Kisspeptin protein in the hypothalamus. The expression of Kisspeptin was detected by immunofluorescence. Blue represents DAPI nuclear staining, and red represents Kisspeptin immunofluorescence staining. control: control group, stress: restraint stress group. 3V, Third ventricle. n=3.

mechanism in the center? Studies have found that the Kisspeptin neurons in the ARC region of the hypothalamus may be a bridge between the HPA axis and the energy metabolism and HPG axis (21, 22).

Kisspeptin is expressed in the placenta, hypothalamus, pituitary gland and gonads (23, 24), and previous studies have confirmed its role in reproductive function. KiSS1/KiSS1R gene inactivation mutations can lead to idiopathic hypogonadotropic hypogonadism (IHH) (25). Animal experiments also confirmed the expression of Kiss1R in GnRH neurons of mice (26). GnRH antagonists were injected into the lateral ventricle of adult mice, and it was observed that the effect of Kisspeptin in promoting the release of LH was inhibited. While the expression of Kiss1 in the hypothalamus is reduced, a disordered estrus cycle also appears (27). The above studies have shown that Kisspeptin plays an important role in the physiological function of GnRH, pubertal development and reproductive function, and is an important factor in maintaining the HPG axis (9).

In addition to its role in the reproductive system (28), since Kisspeptin neurons are expressed in the arcuate nucleus, attention is also paid on its role in energy metabolism in recent years. There are leptin receptors on Kisspeptin neurons. It was found that in low leptin animal models the expression of Kisspeptin decreased. In case of fasting, GnRH release can be reduced, leading to hypogonadotropic hypogonadism, and a decrease in the expression of Kiss1 gene and Kisspeptin protein, and exogenous supplementation of Kisspeptin can improve fasting low levels of gonadotropins. In addition, Kisspeptin is expressed in many peripheral tissues (including pituitary, pancreas, and adipose tissue) related to energy balance and reproduction (29, 30). Kisspeptin is also believed to affect the secretion of metabolic hormones, including aldosterone, adiponectin, insulin, growth hormone, oxytocin, and prolactin (31, 32). Hypothalamic AMPK signaling plays a key role in the metabolic control of puberty, acting *via* a repressive modulation of ARC Kiss1 neurons in conditions of negative energy balance (33). All these observations suggest that Kisspeptin has a potential connection between metabolic state and reproductive function.

Recently, some scholars reported that the use of continuous light stimulation or day and night intervention in rats, immunohistochemistry found that the expression of kisspeptin in the hypothalamus decreased (34), suggesting that the expression of kisspeptin in the hypothalamus will be inhibited under stress. The reproductive dysfunction caused by stress may be related to the inhibition of Kisspeptin neurons in ARC (35, 36).

In addition, glucocorticoid receptor (GR) is expressed on the mouse hypothalamic Kisspeptin neuron cells (11), while the expression of Kisspeptin mRNA in the hypothalamus of female mice is reduced under different stress conditions (31), and our previous studies have also confirmed that dexamethasone can inhibit the transcriptional expression of Kiss1 mRNA and the expression level of Kisspeptin protein in hypothalamic GT1-7 neuronal cells (GnRH and Kisspeptin are both expressed and secreted), and the glucocorticoid receptor blocker RU486 can antagonize this effect. These findings suggest that kisspeptin neurons in the hypothalamus may be a new and important central target that stress affects energy metabolism and reproductive function.

We confirmed in this study that restraint stress can lead to changes in food intake and weight, and lead to disorders of reproductive function, while restraint stress increases cortisol levels. However, it is not clear whether Kisspeptin is involved in the central effect of stress. Therefore, in this study, we used the Cre-loxp system to construct Kiss1 neuron-specific GR knockout mice to further explore whether restraint stress affects energy metabolism and reproductive function, and whether GR plays a role in Kisspeptin neurons.

In this study, we first constructed Kisspeptin neuron-specific GR knockout mice, and tested the role of GR signals on Kisspeptin neurons in the mouse HPA axis in the rhythm of mouse serum cortisol. We collected morning and afternoon blood samples to assess the rhythm of cortisol levels. We found that compared with control GRflox/flox/Kiss1Cre mice, the trough and peak values of serum cortisol in female GRflox/flox/Kiss1Cre+ mice were not significantly different, suggesting that the GR signaling pathway on Kisspeptin neurons is more effective than normal HPA in male mice. The axis may not be a critical signal. In female KGRKO mice, they showed different HPA axis phenotypes. Compared with the control GRflox/flox/Kiss1Cre-mice, female GRflox/flox/Kiss1Cre++ mice had a significantly higher morning serum cortisol trough, but no significant difference in the afternoon serum cortisol peak.

In addition, we also compared the changes of stress on the body weight of KGRKO mice. We found that the body weight of KGRKO restraint stress mice was significantly lower than that of KGRKO mice. Compared with the restraint stress group, the weight loss of the KGRKO+ restraint stress group was reduced. Although KGRKO mice still showed weight loss after restraint stress, compared with control mice, the degree of base body weight loss was less than that of control mice after restraint stress. It is suggested that GR on Kisspeptin neurons is not a key part in the change of energy metabolism caused by restraint stress, but the influence of restraint stress on energy metabolism may partly act through GR on Kisspeptin neurons. In terms of reproductive function, the restraint stress group and KGRKO+ restraint stress group showed loss of preestrus or prolonged estrus cycle. The serum LH and FSH of the KGRKO+ restraint stress group decreased compared with the KGRKO group, and the difference was statistically significant. The above results suggest that restraint stress may partially affect energy metabolism through the GR signaling pathway on Kisspeptin neurons, and its specific mechanism needs to be confirmed by further studies.

In conclusion, this study confirmed that chronic restraint stress has a negative effect on the body's energy metabolism and reproductive function, which is reflected in the weight gain and decrease of mice after chronic restraint stress, as well as reproductive dysfunction. Our further study confirmed that chronic restraint stress caused an increase in cortisol levels and a down-regulation of Kiss1 gene transcription in the hypothalamus. Finally, by constructing Kisspeptin neuron-specific GR knockout mice, we confirmed that chronic restraint stress may partially affect energy metabolism through the GR signaling pathway on Kisspeptin neurons. However, how chronic restraint stress affects peripheral energy metabolism and reproductive function through hypothalamic kisspeptin. In addition to kisspeptin, whether there are other central mechanisms involved, these issues need to be further studied.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Experimental Animal Ethics Committee of Fujian Medical University.

AUTHOR CONTRIBUTIONS

YH, JW, and GC conceptualized and designed these studies, performed them, and wrote the manuscript. YH contributed through data analyses, data interpretation, and manuscript preparation. All authors contributed to manuscript revision and read and approved the submitted version.

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Polycystic Ovary Syndrome Phenotype D Versus Functional Hypothalamic Amenorrhea With Polycystic Ovarian Morphology: A Retrospective Study About a Frequent Differential Diagnosis

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The two most frequent causes of secondary amenorrhea are polycystic ovary syndrome (PCOS) and functional hypothalamic amenorrhea (FHA). Despite several studies showing differences in hormonal profile between these groups, the differential diagnosis remains challenging, in particular between FHA women with polycystic ovarian morphology (FHA-PCOM) and PCOS patients without hyperandrogenism (phenotype D, PCOS-D). In a retrospective case-control study, 58 clearly defined patients with FHA-PCOM were compared to 58 PCOS-D patients, matched 1:1 for age and BMI. Significantly higher levels of LH, estradiol, testosterone, and a higher luteinizing hormone (LH): follicle stimulating hormone (FSH) ratio as well as lower sexual hormone binding globulin (SHBG) levels were found in PCOS-D patients ($p < 0.05$). Optimized cut-off values for the prediction of FHA-PCOM were calculated by the Youden index. The highest sensitivity was found for an estradiol serum level <37.5 pg/mL (84.5%, 95% confidence interval, CI: 72.6-92.6), whereas a LH : FSH ratio <0.96 had the highest specificity (94.8, 95% CI: 85.6-98.9). A linear discriminant analysis including testosterone, SHBG and LH was able to correctly classify 87.9% of FHA-PCOM patients (bootstrap 95% CI: 80.2 - 94.0%). In conclusion, this model including serological parameters could be an easy and reliable tool to distinguish between FHA-PCOM and PCOS-D patients, especially in situations where the clinical profile is not obvious.

Keywords: functional hypothalamic amenorrhea, polycystic ovary syndrome, testosterone, sexual hormone binding globulin, luteinizing hormone, estradiol

INTRODUCTION

Secondary amenorrhea is quite common in women of reproductive age with a prevalence of 3-5%. Notably, the two most frequent causes are polycystic ovary syndrome (PCOS) and functional hypothalamic amenorrhea (FHA) (1). Thus, they are relevant differential diagnoses which can be a challenge for physicians, especially given the fact that a high rate of women with FHA reveal polycystic ovarian morphology (PCOM) of up to nearly 50% (2).

PCOM is one of the key features of PCOS according to the widely used Rotterdam criteria, where two out of three criteria have to be fulfilled which also include clinical and/or serological hyperandrogenism as well as oligo-/anovulation, the latter usually leading to oligo-/amenorrhea (3, 4). Moreover, it is also a major definition criterion according to the Androgen Excess Society (5). In contrast, according to the Endocrine Society, FHA should be defined by a menstrual cycle length persistently exceeding 45 days or amenorrhea >3 months, history of weight loss/vigorous exercise/stress, and the presence of hypogonadotropic hypoestrogenism (1).

Several highly accurate parameters for the differentiation between PCOS and FHA have already been reported and have been reviewed recently (5). These include the body mass index (BMI), levels of luteinizing hormone (LH), androgens, insulin, anti-Müllerian hormone (AMH) and sexual hormone binding globulin (SHBG), the progesterone withdrawal test as well as endometrial thickness as easily applicable tools in clinical routine.

As concluded by Phylactou et al. (5), the exclusion of other reasons for oligo-/amenorrhea is warranted in the definition criteria for both PCOS and FHA and there is no available test that is ultimately discriminating. Moreover, it has been mentioned that these diagnostic uncertainties also make the initial assignment to PCOS or FHA in studies more difficult (6). Thus, a precise definition of PCOS and FHA would be desirable in studies about this specific topic.

The main concern is that one might confuse PCOS without hyperandrogenism (Rotterdam phenotype D; PCOS-D) with FHA with PCOM (FHA-PCOM). However, all previous studies on this topic compared PCOS and FHA without a focus on these special subtypes. We chose two groups of clearly defined cases, namely strictly defined FHA-PCOM patients and age- and BMI-matched women with PCOS-D. Thereby, we aimed to evaluate the most apparent differences in serological patient characteristics and create a simple statistical tool, which should alleviate the differential diagnosis between FHA-PCOM and PCOS-D in more complex situations in the future.

METHODS

This retrospective case-control study was conducted at the Clinical Division of Gynecologic Endocrinology and Reproductive Medicine of the Medical University of Vienna, Austria. From January 2012 to April 2021. Data were included from 58 patients with FHA having PCOM, defined as follows: secondary amenorrhea for at least six months and a negative progestogen challenge test with context of weight loss, insufficient caloric intake, intense physical activity or notion of recent psychological stress, confirmed by a psychologic report. Pregnancy, hypothyroidism, and hyperprolactinemia and any organ-related pituitary dysfunction had to be excluded. The control group consisted of 58 PCOS phenotype D patients diagnosed based on the Rotterdam criteria (4), who had responded well to a progesterone challenge test, and were matched 1:1 by age and BMI for all further analyses in this study. PCOS-D is one of four different phenotypes of PCOS and is also known as "non-hyperandrogenic" PCOS. It is characterized by oligo-/anovulation and PCOM (3). Since the definition of Androgen Excess Society would require hyperandrogenism as a mandatory criterion for PCOS diagnosis (5), the Rotterdam criteria were chosen. This classification has been recently re-visited and validated (3). In all patients, an Aloka Prosound 6 ultrasound machine (Wiener Neudorf, Austria; frequency range 3.0 – 7.5 MHz) was used. PCOM was defined by a follicle number per ovary (FNPO) >12 and/or an ovarian volume ≥10 cm3 and/or an ovarian area ≥5.5 cm^2, according to the recommendations of an international expert panel for ultrasound machine with frequency range less than 8 MHz (7).

The study protocol complies with the declaration of Helsinki and was approved by the Institutional Review Board of the Medical University of Vienna (institutional review board number 1722/2021). Neither written nor verbal informed consent was necessary in retrospective studies according to the Ethics Committee of the Medical University of Vienna.

Parameters Analyzed

As the main outcome parameter, we focused on serum levels of AMH. Additionally, serum levels of total testosterone, androstenedione, SHBG, LH, follicle-stimulating hormone (FSH) and estradiol were also analyzed. The AKIM-software (SAP-based patient management system; SAP Software Solutions Austria, Vienna, Austria) was used for data acquisition.

Blood samples were obtained from a peripheral vein on cycle days 2-5 after bleeding induction with oral dydrogesterone (see below), if possible, or during amenorrhea if no menstruation could be induced with dydrogesterone. All examined blood parameters were determined at the Department of Laboratory Medicine, General Hospital of Vienna, Vienna, Austria according to ISO 15189 quality standards: estradiol, follicle-stimulating hormone (FSH), luteinizing hormone (LH), anti-Mullerian hormone (AMH) and sex hormone-binding globulin (SHBG) were measured by the corresponding Cobas electrochemiluminescence immunoassays (ECLIA) on Cobas e 602 analyzers (Roche, Mannheim, Germany).

The following basic patient characteristics were also included: age at evaluation, body mass index (BMI), gravidity and parity.

Statistical Analysis

Categorical parameters are presented as numbers and frequencies, continuous data as median and their respective interquartile range (IQR). Mann-Whitney U tests were used to

compare independent continuous variables, Wilcoxon rank sum test for dependent continuous variables. Categorical variables between two groups were compared by chi-square or Fisher´s exact test. Receiver operator characteristic (ROC) curves were computed to determine the sensitivity and specificity of serum parameters for FHA-PCOM and help to find optimal cut-off points which were then defined by the Youden index. For these optimized cut-off values, sensitivity, specificity, positive (PPV) and negative predictive values (NPV) with their according 95% confidence intervals (95% CI) are provided. Linear discriminant analyses (LDA) were performed to find linear classifiers separating both groups using FSH, LH, Estradiol, Testosterone, SHBG, and AMH. A confidence interval for the percentage of right classified patients was found by generating 500 bootstrap replicates. Statistical significance was defined by two-sided *P*-values <0.05. Statistical analyses were performed using the open source software "R" (R: The R Project for Statistical Computing).

RESULTS

Basic Patient Characteristics

Due to the matching for age and BMI, these parameters did not differ between women with FHA-PCOM and women with PCOS-D. Concerning hormonal findings, patients with FHA-PCOM revealed significantly higher levels of SHBG. In contrast, significantly higher levels of LH, estradiol, testosterone, androstenedione, DHEAS, and prolactin as well as a higher LH : FSH ratio were found in PCOS-D patients. Details are shown in **Table 1**.

Optimized Cut-Off Values

The ROC curves of the tested serum parameters for FHA-PCOM are shown in the **Supplementary Figure 1**. Only estradiol, testosterone, SHBG, LH, and the LH : FSH ratio were found to be significantly predictive for FHA-PCOM ($p< 0.05$). **Table 2** shows the optimized thresholds for these values calculated by the Youden index. The highest sensitivity was found for estradiol <37.5 pg/mL (84.5%, 95% CI: 72.58-92.65), whereas as a LH : FSH ratio <0.96 had the highest specificity (94.8, 95% CI: 85.6-98.9).

Linear Discriminant Models

In separate linear discriminant models using only one feature, the rates of correctly classified patients were 81.7% for estradiol, 81.7% for testosterone, 71.5% for SHBG, 71.3% for LH, 68.8% for LH : FSH ratio, 55.0% for FSH, and 56.6% for AMH. In **Table 3**, results of the linear discriminant analyses are shown. A linear discriminant analysis incorporating all serologic features, namely estradiol, testosterone, SHBG, LH, the LH : FSH ratio, FSH, and AMH ("full model"), correctly classified 92.9% of the patients (bootstrap 95% CI: 85.3 - 98.2%).

In clinical routine, easily applicable tools must be available. Thus, two "reduced models" were calculated. The "reduced model 1" included only the three strongest parameters estradiol, testosterone and SHBG and was able to correctly classify 91.3% of the patients (bootstrap 95% CI: 84.5 - 96.6%). However, one of the main criteria to assign patients in either the FHA-PCOM or PCOS-D group was whether menstruation could be induced by a gestagen withdrawal test (see Methods Section), which is known to be strongly correlated to serum estradiol levels. Since the aim was to generate a model using serum parameters which should help to distinguish between the two entities in more complex situations, a "reduced model 2" without estradiol was calculated. This model included the features testosterone, SHBG, and LH and correctly classified 87.9% of patients (bootstrap 95% CI: 80.2 - 94.0%).

The ROC curves for all these three models are provided in **Figure 1**.

Practical Application of the Linear Discriminant Models

The "reduced model 2" (**Table 2**) is simple to use. The linear combination of the weighted features is either ≤0, whereby the patient belongs to the group of FHA-PCOM patients, or >0, whereby the patient belongs to the group of patients of PCOS-D. The following formula must be used: (7.05*testosterone ng/mL) – (0.005*SHBG nmol/L) + (0.117*LH mIU/mL) - 2.463. A scatter plot showing the results of this calculation for women with FHA-PCOM and women with PCOS-D is provided in **Figure 2**. The following predictive values for FHA-PCOM using the mentioned cut-off point of ≤0 were: sensitivity 87.9% (95% CI: 76.7-95.0), specificity 89.7% (95% CI: 78.8-96.1), positive predictive value

TABLE 1 | Basic patient characteristics and results of hormonal testing in FHA-PCOM and PCOS-D patients. Data are presented as mean ± SD.

	FHA-PCOM	PCOS-D	p
Age (years)	25.5 ± 4.7	25.5 ± 4.7	1.000
BMI (kg/m^2)	26.4 ± 6.3	26.3 ± 6.2	0.983
TSH (IU/mL)	1.7 ± 0.7	2.0 ± 1.0	0.053
FSH (mIU/mL)	5.2 ± 2.1	5.1 ± 1.9	0.930
LH (mIU/mL)	3.6 ± 3.1	8.8 ± 5.6	<0.001
LH : FSH ratio	0.7 ± 0.5	1.7 ± 1.0	<0.001
Prolactin (ng/mL)	10.2 ± 4.1	13.5 ± 7.7	0.036
Estradiol (pg/mL)	23.0 ± 14.1	53.3 ± 19.4	<0.001
Testosterone (ng/mL)	0.22 ± 0.12	0.38 ± 0.09	<0.001
Androstenedione (ng/mL)	2.0 ± 1.1	2.7 ± 0.9	0.003
DHEAS (µg/mL)	2.2 ± 1.1	2.5 ± 0.8	0.048
SHBG (nmol/L)	81.8 ± 41.8	49.8 ± 37.4	<0.001
AMH (ng/mL)	6.9 ± 3.8	8.4 ± 5.1	0.071

TABLE 2 | Optimized cut-off values for FHA-PCOM.

Parameter	Statistical method	Optimized cut-off value	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)	p
Estradiol	Youden index	<37.5 pg/mL	84.48 (72.58-92.65)	82.76 (70.57-91.41)	83.05 (73.39-89.69)	84.21 (74.31-90.77)	<0.001
Testosterone	Youden index	<0.31 ng/mL	79.31 (66.65-88.83)	86.21 (74.21-93.85)	85.19 (74.89-91.73)	80.65 (71.36-87.45)	<0.001
SHBG	Youden index	>61.4 nmol/L	68.97 (55.46-80.46)	79.31 (66.65-88.83)	76.92 (66.18-85.03)	71.88 (63.01-79.31)	<0.001
LH	Youden index	<4.7 mIU/mL	74.14 (60.69-84.74)	77.59 (64.73-87.49)	76.79 (66.68-84.54)	75.00 (65.51-82.57)	<0.001
LH: FSH ratio	Youden index	<0.96	72.41 (59.10-83.34)	94.83 (85.62-98.92)	93.33 (82.14-97.71)	77.46 (96.28-83.97)	<0.001

All data are provided as %; PPV, positive predictive value; NPV, negative predictive value.

89.5% (95% CI: 79.8-94.8), and negative predictive value 88.1% (95% CI: 78.7-93.7; p< 0.001).

In case of patient number 1, who belongs to the group of women with FHA-PCOM, the patient had a testosterone level of 0.3 ng/mL, SHBG of 69.1 nmol/L and LH of 2.1 mIU/mL. The linear classifier is computed as:

$$(7.05 * 0.30) - (0.005 * 69.1) + (0.117 * 2.1) - 2.463 = -0.4478$$

The weighted sum is below zero and therefore the patient belongs to the group of FHA-PCOM women.

In contrast, patient number 2 who belongs to the group of PCOS-D women, revealed the following serum parameters: testosterone 0.44 ng/mL, SHBG 131.90 nmol/L, and LH 17.2 mIU/mL. In this case, the linear classifier is computed as:

$$(7.05 * 0.44) - (0.005 * 131.9) + (0.117 * 17.2) - 2.463 = 1.9919$$

The weighted sum is greater than zero and, thus, the patient is allocated to the group of women with PCOS-D.

DISCUSSION

To distinguish women with FHA-PCOM from women with PCOS-D, the present study revealed that the following parameters would be useful: testosterone, LH, the LH : FSH ratio, and SHBG. Using optimized cut-off values calculated by the Youden index, the sensitivity ranged from about 72% to about 79% (**Table 2**). Including these features in linear discriminant analyses, even a "reduced model" using only a minority of accurate parameters, 87.9% of patients could be classified correctly.

We decided to exclude estradiol from our discriminating variables, although its level was significantly lower in women with FHA-PCOM than in PCOS-D patients, a fact that has been reported many times (8). The reason for this is that in our study, a progesterone withdrawal test was used to assign patients in either the FHA-PCOM or PCOS-D group. This was done to define the two groups in the best way possible. Therefore, highly significantly declined estradiol levels were observed in our FHA group, but this result was obviously biased, due to our methodical approach. Another reason for not using estradiol is that previous articles on women with FHA (2, 9) suggested intermittent estrogen production and, thus, levels within the normal range. Therefore, the data about estradiol in our model with well-defined cases should not be used to better distinguish between patients, where assignment to one or the other group is not similarly obvious.

This leads to the question of how patients were assigned to the groups. Any methodical approach to this question can be considered problematic. However, strict definition criteria of FHA-PCOM were defined: in addition to the negative progesterone challenge test, a cause for FHA had to be evident, namely weight loss, insufficient caloric intake, intense physical activity or notion of recent psychological stress. Thus, it seems very likely that all women classified as "FHA-PCOM" actually suffered from this entity. However, one could see the definition of PCOS- D as problematic and might assume that some FHA-PCOM patients may have been allocated to this group incorrectly. We consider these circumstances as a study limitation. However, based on the strict criteria, we believe that we have been able to define the groups in the best way possible.

Apart from estradiol, testosterone showed the highest sensitivity for the diagnosis of FHA-PCOM, with an optimized cut-off value of 0.31 ng/mL. This was associated with a high specificity of about 86%, which was also reflected by the high PPV of 85.2 and NPV of 80.6% (**Table 2**). These results show the importance of testosterone despite the fact that per definition, patients with PCOS-D do not have clinical or serological hyperandrogenemia. Nevertheless, testosterone was significantly

TABLE 3 | Results of the linear discriminant analyses.

Parameter	FHA-PCOM	PCOS-D	Coefficients of linear discriminants		
			Full model	Reduced model 1	Reduced model 2
Estradiol (pg/mL)	23.0 ± 14.1	53.3 ± 19.4	0.039	0.004	
Testosterone (ng/mL)	0.22 ± 0.12	0.38 ± 0.09	4.687	5.108	7.050
SHBG (nmol/L)	81.8 ± 41.8	49.8 ± 37.4	-0.006	-0.009	-0.005
LH (mIU/mL)	3.6 ± 3.1	8.8 ± 5.6	0.012		0.117
LH : FSH ratio (mIU/mL)	0.7 ± 0.5	1.7 ± 1.0	0.414		
FSH (mIU/mL)	5.2 ± 2.1	5.1 ± 1.9	0.060		
AMH (ng/mL)	6.9 ± 3.8	8.4 ± 5.1	0.035		
Constant			-3.637	-2.601	-2.463

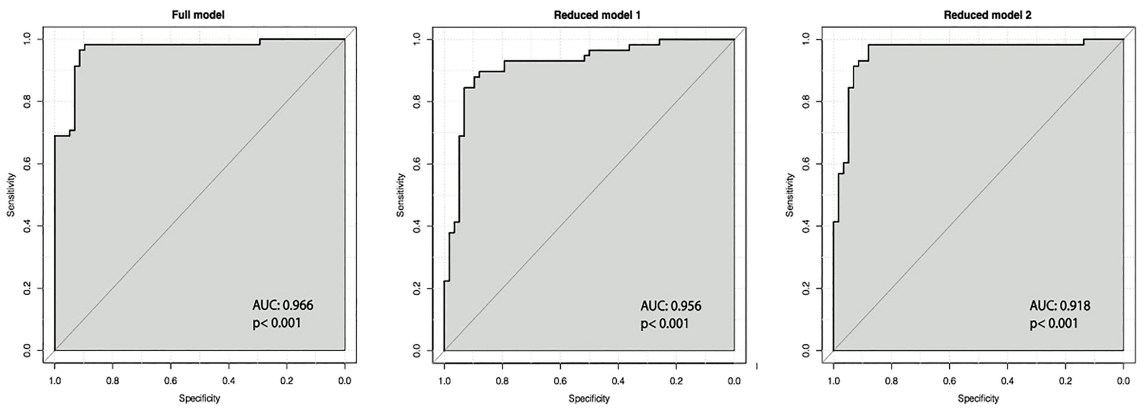

FIGURE 1 | ROC curves for linear discriminant models. For each parameter, the area under the curve (AUC) and the p-value are provided.

higher in PCOS-D patients than in women with FHA-PCOM, although being in the normal range (**Table 1**). Since the ovaries are a main source for testosterone production in women (10) and ovarian function is limited in FHA (2), it seems intuitive that lower testosterone levels are found in FHA patients.

Moreover, in our analysis, the LH : FSH ratio with a cut- off value of <0.96 has been shown to be a strong predictor, very reliably predicting FHA-PCOM for the individual patient with a PPV of 93.3% (**Table 2**). It is known that PCO patients tend to have an increased LH : FSH ratio (11), while on the other hand FHA patients have lower LH levels (2, 9). Together with the significantly lower and also highly predictive LH levels (**Tables 1** and **2**), our results are consistent with the existing literature. One might argue that the LH : FSH ratio of 1.7 found in our PCOS patients would not be typical. However, it has been reported that testosterone levels would be positively correlated with the LH : FSH ratio (12). Since only PCOS-D women without hyperandrogenism were included, the comparably lower mean LH : FSH ratio would be reasonable.

Contrary to our expectations, the prognostic potential of SHBG, previously reported as a promising parameter to distinguish between FHA and PCOS (6), was comparably moderate. It is known that PCOS patients reveal lower SHBG levels (13). However, a recent analysis by Makollé et al. (2) already showed that women with FHA-PCOM might have a tendency to metabolic aspects of the PCO syndrome. Moreover, in this study, it became evident that patients with FHA-PCOM revealed lower SHBG levels than FHA women without PCOM (2). This could explain why SHBG proved to be weaker in prediction of FHA, although a PPV of approximately 77% and a NPV of 72% can be considered relatively reliable for a single parameter. **Table 1** displays wide variations of SHBG levels in both groups. It should be mentioned that women with FHA and underweight and/or eating disorders are known to have very high SHBG levels (14, 15).

In contrast to all these parameters, the ROC analyses showed that AMH was not predictive (**Supplementary Figure 1**). Recently, it has been described that especially women with FHA-PCOM tended to have high AMH levels, compared to non-PCOM FHA patients (2), which explained previous findings (9, 16). It has been hypothesized that FHA-PCOM patients initially exhibit components of PCOS before subsequently developing FHA due to weight loss, insufficient caloric intake, stress, or excessive exercise. Conclusive for this hypothesis is also a higher body mass index (BMI) and lower levels of sex hormone binding globulin (SHBG) in FHA-PCOM patients with PCOM compared to non-PCOM FHA women (2).

For this reason, AMH was not included in the calculations of the reduced linear discriminant models (**Table 3**). We believe that especially the reduced model 2, which did not include estradiol due to the above-mentioned methodical considerations, might be the most relevant and clinically applicable one. This model can be used to enable a well-founded differential diagnosis in less clearly defined cases. It offers equally high PPV and NPV of 89.5% and 88.1%, respectively. The majority of FHA-PCOM cases could be classified correctly (87.9%), which is also underlined by the small confidence interval in the bootstrap analysis (80.2 - 94.0%). In absence of any other clear criteria to distinguish the two entities from each other, we consider this approach helpful. We are aware of the fact that future studies are needed to clarify whether the model is correct and probably adjust it.

Concerning possible study limitations, the above-mentioned definition criteria of the two groups must be considered once more. In addition to the already mentioned considerations, one might wonder whether the included FHA population consisted in fact of PCOS-D patients who had hypogonadotropic stress and were recruited after partial recovery. Although this cannot be ruled out completely, it has already been shown that women with well-defined FHA-PCOM were not at an increased risk for developing PCOS in the course of pulsatile GnRH therapy (17). Moreover, only in a minority of FHA women, PCOS becomes unmasked by pulsatile GnRH treatment (18). Thus, we assume that it is unlikely that there was a majority of PCOS-D patients who were unintentionally allocated to the FHA-PCOM group.

Although the model is proposedly useful to help clinicians with an easy tool to distinguish between FHA-PCOM and

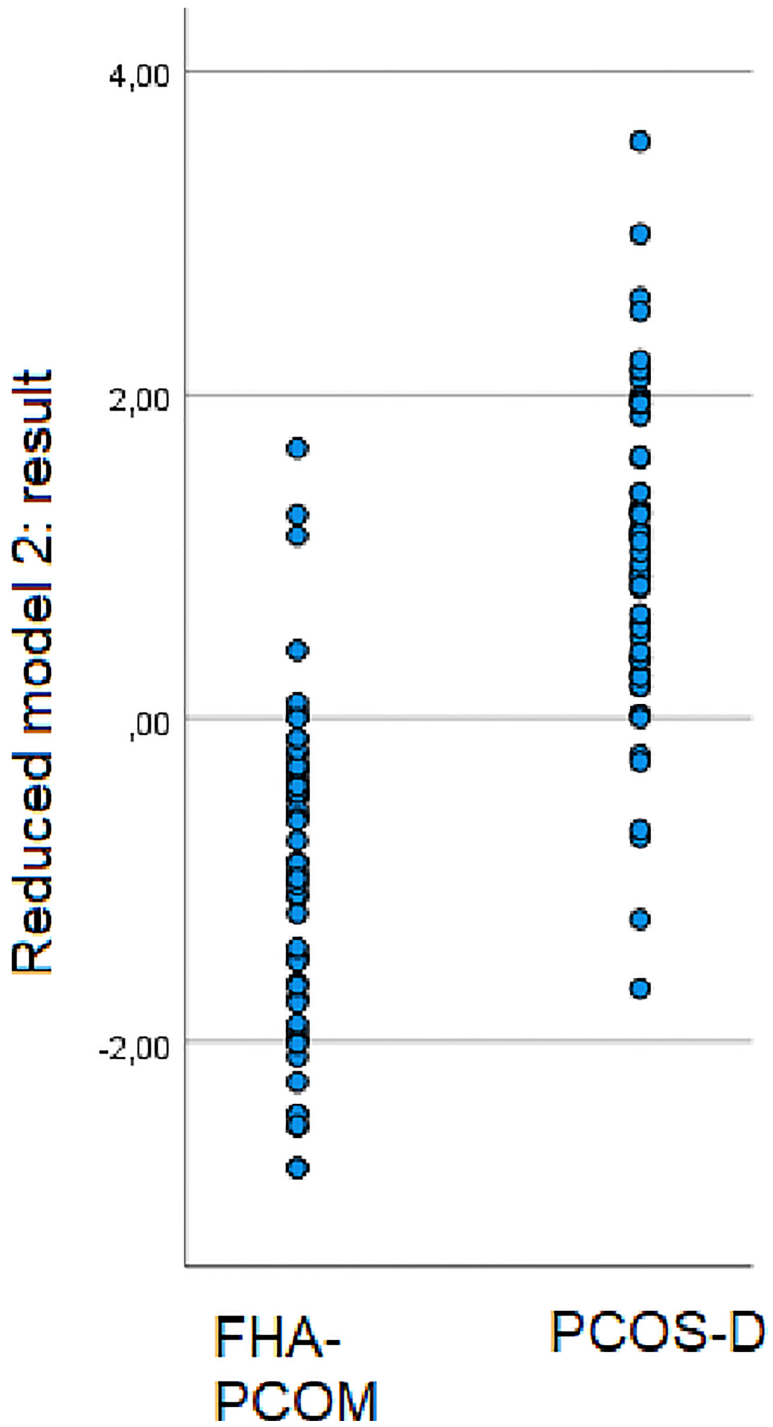

FIGURE 2 | The reduced linear discriminant analysis includes testosterone, SHBG and LH as predictive parameters for FHA-PCOM. The scatter plot shows the results of the formula used "(7.05*testosterone ng/mL) – (0.005*SHBG nmol/L) + (0.117*LH mIU/mL) - 2.463" for women with FHA-PCOM and PCOS-D.

PCOS-D in less clearly defined cases, it may not be transferable to all patients in every case. In addition, the retrospective study design and the small sample size must certainly be mentioned as study limitations. On the other hand, to our knowledge, this is the first study to attempt to discriminate between two groups which are likely very difficult to distinguish from each other, namely FHA-PCOM and PCOS-D, under optimally defined criteria. Moreover, one might argue that the mean BMI of

about 26 kg/m^2 is unusual for women with FHA who often suffer from underweight and eating disorders. It should be emphasized that we matched both groups on BMI, which obviously resulted to select FHA-PCOM patients with normal or slightly elevated BMI. It is precisely those patients who are at risk to be diagnosed PCOS-D.,Since we chose to use strict criteria for the definition of FHA, many women with intense physical activity were included. These often reveal a normal BMI due to the high muscle mass. In addition, the notion of recent psychological stress in FHA women is also not necessarily associated with a low BMI. Nonetheless, this circumstance should be considered a minor study limitation. In addition, the mean AMH levels of 6.9 ng/mL could be considered high for FHA patients. However, similar levels have been reported previously. For example, in a cluster analysis, 48% of FHA patients revealed PCOM. In a cluster with 70% of women with PCOM, the median AMH was 60.6 pmol/L, which is equal to 8.48 ng/mL (9). Many other publications showed normal or increased AMH levels in FHA patients despite low FSH and LH levels (9, 19, 20). Further studies even reported significant differences in AMH levels comparing FHA patients to controls (16, 21, 22).

In conclusion, clinical and serological differentiation between FHA-PCOM and PCOS-D can be challenging. A combination of low testosterone levels, a low LH : FSH ratio, and a higher SHBG level yielded the strongest predictive value for FHA, compared to any of the most discriminant variables used alone. The formula "(7.05*testosterone ng/mL) – (0.005*SHBG nmol/L) + (0.117*LH mIU/mL) - 2.463" can be used as an easy tool for this differential diagnosis. Further studies would be desirable to shed more light on this challenging topic.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Medical University of Vienna (institutional review board number 1722/2021). Written informed consent was not required for this study, in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

KB: protocol/project development, data collection or management, data analysis, manuscript writing/editing, final proof-reading. DD: protocol/project development, data management, data analysis, manuscript writing/editing, final proof-reading. RS: protocol/project development, data management, data analysis, manuscript writing/editing, final proof-reading. MH: protocol/project development, data collection or management, manuscript writing/editing, final proof-reading. JB: protocol/project development, data collection, final proof-reading. DM: protocol/project development, data collection or management, final proof-reading. IH: protocol/project development, manuscript writing/editing, final proof-reading. JO: protocol/project development, data collection or management, data analysis, manuscript writing/editing, final proof-reading. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.904706/full#supplementary-material

Supplementary Figure 1 | *ROC curves for FHA-PCOM*. For each parameter, the area under the curve (AUC) and the p-value are provided.

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The Reproductive Outcome of Women with Hypogonadotropic Hypogonadism in IVF

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Objective: The purpose of this study was to evaluate the reproductive outcome of patients with hypogonadotropic hypogonadism (HH) receiving *in vitro* fertilization and embryo transfer (IVF-ET).

Methods: The reproductive outcome of 81 HH patients and 112 controls who underwent oocyte retrieval was evaluated retrospectively in the Center for Reproductive Medicine of Peking University Third Hospital from 2010 to 2019.

Results: The basic levels of follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), androstenedione (A) and prolactin (PRL) were significantly lower in the HH group than the control group. Although the HH patients required a significantly longer stimulation and higher gonadotropin (Gn) doses than the control patients, the total number of oocytes retrieved, fertilized embryos, two pronuclear (2PN) embryos, transferable embryos, fertilization and 2PN rates were comparable between the two groups. Although the live birth rate (LBR) of the first fresh cycle was higher in the control group than the HH group, there was no statistical significance. Then we further divided HH patients into two subgroups according to the etiology. Forty-one cases were termed as congenital HH (CHH), while the other 40 cases were termed as acquired HH (AHH), the latter includes functional hypothalamic amenorrhea (FHA) and pituitary HH (PHH). Our results showed that there were no significant differences in basic clinical characteristics and IVF parameters between the two groups. In the HH group, a total of 119 oocyte retrieval cycles were carried out and they responded adequately to ovulation induction. Urinary human menopausal gonadotropin (HMG) was used alone in 90 cycles while combination of HMG and recombinant human follicle stimulating hormone (rFSH) in the other 29 cycles. There were no significant differences in IVF-related parameters between the two groups. The conservative cumulative live birth rates (CLBRs) after the first, the second and ≥third cycles were 43.21%, 58.02% and 60.49%, respectively, while the corresponding optimal CLBRs were 43.21%, 68.45% and 74.19%. The preterm birth (PTB) rates of singletons and twin pregnancy in HH patients were 8.33% (3/36) and 30.77% (4/13), respectively.

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Conclusion: IVF-ET is an effective treatment for HH patients with infertility and patients can get satisfactory pregnancy outcomes.

Keywords: hypogonadotropic hypogonadism, *in vitro* fertilization and embryo transfer, infertility, cumulative live birth rate, anovulation

INTRODUCTION

Hypogonadotropic hypogonadism (HH) is caused by deficiencies in hypothalamic endogenous gonadotropin-releasing hormone (GnRH) release or pituitary gonadotropin (Gn) secretion, leading to an imbalance in the hypothalamic-pituitary-gonadal (HPG) axis and diminished ovarian function. From a reproductive health perspective, HH is classified as WHO type I ovulatory disorder and the diagnosis of HH should differentiate from other forms of primary or secondary amenorrhea. The basic approach is an assessment of the sex hormones, and other diagnostic tools include but are not restricted to pelvic examination, abdominal or transvaginal ultrasound, progestin challenge, cerebral magnetic resonance imaging (MRI) scan, detailed personal history collection with a focus on diet, eating disorders, exercise, weight, development, menstruation and mental distress, bone mineral density test, detection of thyroid hormones, prolactin (PRL) determination, and GnRH stimulation test. The causes of HH can be congenital or acquired, and according to the pathophysiology, HH is categorized into congenital HH (CHH), pituitary HH (PHH), and functional hypothalamic amenorrhea (FHA). CHH is caused by deficient production, secretion or action of GnRH, and characterized by incomplete or absent puberty and infertility, which can present solely as congenital GnRH deficiency or be associated with other developmental anomalies (1). When associated with anosmia or hyposmia, CHH is termed as Kallmann syndrome, which results from abnormal embryonic migration of GnRH neurons from their origin in the olfactory placode to the forebrain (2). The diagnosis of CHH is necessarily one of exclusion, since it requires acquired, functional or structural conditions to be ruled out. CHH is genetically a heterogeneous disorder with identified X-linked, autosomal dominant, and autosomal recessive patterns of inheritance. With the development of next-generation sequencing (NGS) techniques, pathogenic variants in more than 50 genes have been identified in CHH (3). However, each gene linked to CHH only seems to underpin a small percentage of total patients, so we are still far from achieving a comprehensive understanding of the genetic basis of CHH. Patients have generally not benefited from advances in genetics in respect of novel therapies (4). FHA is a form of chronic anovulation that is not due to identifiable organic causes. Several etiologic factors have been well described for FHA, including intense or frequent exercise, weight loss, psychological stress, and psychological disturbance (5, 6), while in many, no eliciting factors can be identified. Importantly, even in those for whom causative factors have been suggested, the precise mechanism of disruption of GnRH secretion has not been elucidated. Previous reports identified several rare genetic variants in genes related to GnRH deficiency in FHA patients. Therefore, patients with FHA may have a genetic basis partially in common with idiopathic CHH and the total load of mutations in genes related to GnRH action might be less in FHA than that in idiopathic CHH (7). Common causes related with PHH includes infiltrative or infectious pituitary lesions, pituitary apoplexy, and radiation.

Due to the long-term deficiency stimulation of Gn, women with HH show symptoms of hypoestrogenism, hypoplasia of uterus, ovulation inefficiency, and amenorrhea. Of note, the management of HH with each category requires different approaches. The principal target of treatment for CHH is supplying exogenous Gn or sex steroid hormones depending on the timing and aim of treatment whether it is pubertal induction, general health or fertility (8). For FHA patients, we need to find the predisposing factors, provide psychological support and improve energy balance. In addition to the above management, the management of women with PHH is distinctive from CHH because they require extensive multidisciplinary input with endocrinologists to address the cause and the different pituitary functions affected (9). Due to estrogen deficiency, the health in HH patients is disturbed in several aspects including their skeletal system, cardiovascular system and mental problems (10). Herein we should also pay attention to its long-term complications.

Anovulation and infertility are common complaints among women with HH who require hormonal therapeutic intervention. For patients with fertility requirements, artificial hormone replacement (HRT) with estrogen and progesterone are primarily applied to promote uterus development. Then, when the size of uterus is normal, ovarian stimulation with Gn or pulsatile GnRH is used to induce ovulation. Pulsatile GnRH treatment has been utilized for the management of women with hypothalamic HH suffering from infertility. However, pulsatile GnRH requires near perfect compliance and close monitoring. In addition, the use of a portable pump injection device and the need to inject subcutaneously or intramuscularly has been regarded as a disadvantage (11). In clinical practice, the more commonly used ovulation induction procedure for patients with HH is daily low-dose injections of Gn, which is applicable to all types of HH. HH women do not have endogenous luteinizing hormone (LH), therefore, Gn with an LH component is required. The development of urinary-derived Gn (human menopausal gonadotropin, HMG) containing both follicle stimulating hormone (FSH) and LH a few decades ago paved the way to replacing the absent endogenous hormones. The prognosis for inducing ovulation with HMG in most of the HH patients is favorable through guided coitus or intrauterine insemination (IUI). However, a

few of patients may present with ovulation induction failure or multiple ovarian follicles development leading to ovarian hyperstimulation syndrome (OHSS). Besides, some other couples are unable to be pregnant naturally due to fallopian tube, male factors or other causes.

Assisted reproductive technology (ART), including *in vitro* fertilization (IVF), has developed in recent years and is a better effective choice for the patients who fail to be pregnant through ovulation induction. However, the low incidence of HH makes it difficult to evaluate the reproductive outcome of IVF in women with HH. Limited studies have shown that, all categories of HH are considered as a whole group to compare IVF characteristics with control group (12–14). However, whether there are differences in IVF characteristics among patients with different types of HH have not been reported. Due to the long-term deficiency of endogenous estrogen stimulation, how HH patients respond to exogenous Gn and their pregnancy outcomes especially the live birth rate (LBR) are still unknown. Besides, since the uterine development may be impaired after long-term amenorrhea, whether HH patients have increased pregnancy risks, such as late pregnancy abortion and preterm labor, is also unclear. In this study, we report a retrospective analysis performed on 81 patients with HH and 112 controls in the Center for Reproductive Medicine of Peking University Third Hospital with the aim to analyze the clinical IVF outcomes of women with HH. Furthermore, we also made a detailed analysis on IVF outcomes among patients with different types of HH.

MATERIALS AND METHODS

Patients

A total of 81 women with HH who underwent oocyte retrieval in the Reproductive Center of Peking University Third Hospital from 2010 to 2019 were included. The diagnosis of HH was based on the primary or secondary amenorrhea, absence of withdrawal bleeding following a progestin challenge test, and normal or low levels of serum FSH and LH (13). Other causes of amenorrhea, such as polycystic ovary syndrome, uterine disorder or ovarian dysfunction were excluded. Combined with the patient's medical history and auxiliary examination results, 41 patients were classified as CHH group, 31 patients as FHA group and the other 9 patients as PHH group. Here, due to the small number of patients with PHH, we grouped them with FHA and termed as acquired HH (AHH). Most patients experienced previous multiple ovulation induction with or without IUI or IVF cycles and failed to get pregnancy, and some other patients received IVF because of oviduct factors. The major cause for IVF was the failure to be pregnant after guided coitus or IUI with ovulation induction. Fourteen of them underwent intracytoplasmic sperm injection (ICSI) for coexisting male factor infertility. As a control group who underwent IVF with matched background characteristics during the same period, 112 women diagnosed with tubal factor or male factor infertility were also evaluated.

Controlled Ovarian Stimulation Protocols

Ovulation Induction Protocol for HH Patients

At least 3 cycles of HRT were prescribed to promote uterus development before initiating the COS on the second or third day of menstruation. Then they underwent a total of 119 oocyte retrieval cycles. Urinary gonadotropin for injection (HMG, Livzon, China) was used alone in 90 cycles while combination of HMG and recombinant human FSH for injection (rFSH, Gonal-F, Merck Serono, Germany) in the other 29 cycles. Serum levels of estradiol (E2), progesterone (P), FSH, and LH were detected on the second day of menstruation and the monitoring was performed using ultrasound, and then Gn dosage was adjusted based on the individual patient's ovarian response.

Ovulation Induction Protocol for Control Patients

In the control group, GnRH agonist (GnRH-a) or antagonist (GnRH-ant) regimes were used. In the long or ultra-long GnRH-a protocol, Triptorelin Acetate (Diphereline, Ipsen, France) with a dose of 1.8 mg or 3.75 mg was administered in the mid-luteal phase or the menstrual period of the previous menstrual cycle. Fourteen or 28 days later, rFSH was administered after pituitary suppression was demonstrated. In GnRH-ant regime, rFSH was administered on the second day of menstruation and pituitary suppression was managed with Cetrorelix (Cetrotide, Merck Serono, Germany) starting on the sixth or seventh day of the cycle according to the follicular growth (when the leading follicle reached a diameter of 13-14 mm).

For all the subjects, 10000 IU human chorionic gonadotropin (HCG, Livzon, China) or 250 ug recombinant human choriogonadotropin alfa solution for injection (rHCG, Ovidral, Merck Serono, Germany) were triggered when at least the diameter of 3 dominant follicles reached 18 mm and oocyte retrieval was usually performed with transvaginal ultrasound 36 h after HCG injection. Conventional IVF or ICSI was performed depending on the semen analysis on the day. Two embryos or one blastocyst transfer was performed on the day of 3 or 5 later after oocyte retrieval. Serum β-HCG was measured 14 days following embryo or blastocyst transfer. Clinical pregnancy was defined as a positive pregnancy blood test followed by the presence of gestational sac on transvaginal ultrasound 30 days after the embryo or blastocyst transfer. Luteal support was maintained until 10 weeks of gestation if conception occurred.

Statistical Analysis

R4.0.3 statistical software was used for statistical analysis. The quantitative data were given as means ± standard deviations or as medians ± quartile (25% quantile, 75% quantile), as appropriate. Qualitative data were expressed by count and percentage. Age was compared between the two groups using an independent sample T test. Wilcox test was employed to analyze the differences of IVF parameters between HMG and combination groups. Chi-square test was used to compare the rates or proportions. A P value of less than 0.05 (2-sided significance testing) was considered to be statistically significant.

RESULTS

Clinical Characteristics and IVF Outcomes of Patients

The characteristics of the 81 women with HH and 112 controls are shown in **Table 1**. There were no differences in age or body mass index (BMI) between the HH group and the control group. But the basic levels of FSH, LH, E2, androstenedione (A), and PRL were significantly lower in the HH group than those in the control group.

We have compared the IVF characteristics of the first fresh cycle of HH patients with control group. And the outcomes are detailed in **Table 2**. The HH patients required a significantly longer stimulation and higher Gn doses than the control patients. Although larger amounts of Gn were used, the serum LH and P levels on HCG day were still significantly lower in HH patients than that in control patients. E2 level was also higher in the control group, but the difference was not statistically significant. The total number of oocytes retrieved, fertilized embryos, two pronuclear (2PN) embryos, transferable embryos, fertilization rate, and 2PN rate were comparable between the two groups. In the HH groups, 63 cases received fresh cycles transplantation and 23 cases got a live birth, while in the control group, 42 cases delivered among the 88 patients who underwent their fresh transplantation cycle. Although the live birth rate (LBR) of the fresh cycle was higher in the control group than that in the HH group, there was no statistical significance. In our study, GnRH-a regime was used in 59 patients and GnRH-ant regimes was used in 53 patients. Then we further compared HH group with the two regimes respectively and found that the total number of oocytes retrieved, fertilized embryos and 2PN embryos were higher in the GnRH-a regime group than that in the HH patients, but the number of transferable embryos, fertilization and 2PN rate were comparable between the two groups, while compared with the GnRH-ant regime group, no differences in IVF outcomes were observed. Moreover, a larger amount of Gn and a longer duration of ovarian stimulation were necessary in HH patients regardless of whether GnRH-a and GnRH-ant regimes were selected in the control group (**Supplementary Tables 1, 2**).

IVF Outcomes

Then we focused on pregnancy outcomes in patients with HH. A total of 119 oocyte retrieval cycles were carried out in the 81 patients. Among them, 27 patients underwent 2 while another 10 patients underwent 3 oocyte retrieval cycles. Only 1 patient underwent 4 cycles. All the HH patients responded adequately to ovulation induction. Transferable embryos were obtained in 112 cycles, accounting for 94.12%. Only 1 cycle had no oocyte and another 6 had no embryos to transfer after the oocyte retrieval. Embryos were transferred in 92 fresh cycles and frozen in 20 cycles. This decision of embryo frozen was made for 13 cases to prevent the risk of OHSS and no severe OHSS was detected in all patients, and the other 7 patients for elevation of P or personal reasons. According to the medicine for ovulation induction we used, the 119 cycles were divided into two groups, one group used HMG (90 cycles) alone while another group used HMG combined with rFSH (29 cycles). IVF outcomes were compared between the two groups and there were no significant differences in IVF-related parameters between HMG and HMG combined with rFSH groups (**Table 3**).

Cumulative live birth rate (CLBR), which represents the total chance rate of LBRs of each retrieval cycle after all the embryos obtained are transferred, was calculated (15). Conservative CLBR assumed that women who did not return for treatment would not have a live birth, whereas optimal CLBR assumed that these women would have LBRs similar to those for women continuing treatmentcs. In the first cycle of 81 patients, 35 cases were successful in pregnancy and delivery, and the LBR was 43.21%. Twenty-seven cases underwent the second cycle of oocyte retrieval, 12 cases delivered and the LBR was 44.44%. The third cycle for oocyte retrieval was performed in 10 patients, but only one succeed. We had one patient performing the fourth cycle and finally got a live birth. As the number of patients having three or more treatment cycles was small, patients who completed more than three cycles were grouped into one group for analysis. The conservative CLBRs after the first, the second, and ≥ third cycles were 43.21%, 58.02%, and 60.49%, respectively, while the corresponding optimal CLBRs were 43.21%, 68.45%, and 74.19% (**Table 4**).

The definition of preterm birth (PTB) is the delivery prior to 37 weeks. Among the 49 live births, there were 36 singletons, including 33 full-term births, 3 PTBs and 13 twins, including 9 full-term births and 4 PTBs. Only 1 preterm infant delivered at 29 weeks of gestation, and the other 6 delivered at 36 weeks.

Another interesting question was that whether there was a difference in IVF outcomes between patients with different HH categories. Our results showed that there were no significant

TABLE 1 | Comparison of clinical characteristics between hypogonadotropic hypogonadism (HH) and control groups.

Variables	HH group (n=81)	Control (n=112)	*P*
Age (y)	30 (27, 32)	30 (29, 32)	0.669
BMI (kg/m^2)	21.01 (19.53, 23.60)	21.49 (19.58, 23.65)	0.578
Basal serum hormonal level			
FSH (mIU/mL)	1 (0.36, 2.88)	6.71 (5.43, 8)	< 0.001
LH (mIU/mL)	0.3 (0.1, 0.78)	3.36 (2.37, 4.73)	< 0.001
E2 (pmol/L)	82.2 (73.4, 119)	157.42 (121.25, 221.38)	< 0.001
A (nmol/L)	4.54 (3.21, 6.06)	6.3 (4.8, 8.38)	< 0.001
PRL (ng/mL)	5.65 (4.28, 8.68)	12 (8.91, 17.7)	< 0.001

BMI, body mass index; FSH, follicle stimulating hormone; LH, luteinizing hormone; E2, estradiol; A, androstenedione; PRL, prolactin. The data are expressed by the median (25% quantile, 75% quantile), and the comparison between the two groups is performed by Wilcox test.

TABLE 2 | Comparison of cycle characteristics between hypogonadotropic hypogonadism (HH) and control groups.

Variables	HH group (n=81)	Control (n=112)	*P*
Hormone levels on HCG day			
E2 (pmol/L)	6751 (4524.5, 12656)	10251 (5678.75, 13948.75)	0.128
LH (mIU/mL)	0.18 (0.11, 0.56)	1.01 (0.6, 2.32)	< 0.001
P (nmol/L)	1.77 (1.42, 3.09)	2.45 (1.56, 3.57)	0.035
Duration of stimulation (d)	14 (13, 16)	11 (10, 12)	< 0.001
Total amount of Gn injected (IU)	3487.5 (2850, 4500)	2550 (1715.62, 3318.75)	< 0.001
No. of oocytes retrieved	11 (7, 14)	11.5 (8, 17)	0.177
No. of fertilized embryos	9 (5, 11)	8 (5, 12.25)	0.512
No. of 2PN embryos	6.5 (4, 9)	7 (4, 10)	0.334
No. of non-2PN embryos	1 (0, 2)	1 (0, 2)	0.464
Fertilization rate (%)	0.79 (0.64, 0.97)	0.76 (0.62, 0.89)	0.244
2PN rate (%)	0.86 (0.72, 1)	0.86 (0.73, 1)	0.497
No. of transferable embryos	3.5 (2, 8)	4 (2, 8.25)	0.704
LBR (%)	36.5 (23/63)	47.7 (42/88)	0.170

HCG, human chorionic gonadotropin; E2, estradiol; LH, luteinizing hormone; P, progesterone; Gn, gonadotropin; PN, pronuclear; LBR, live birth rate. The data are expressed by the median (25% quantile, 75% quantile), and the comparison between the two groups is performed by Wilcox test and Pearson Chi square test.

differences in basic clinical characteristics and IVF parameters between CHH and AHH groups (**Table 5**). Besides, we also made a comparative analysis between CHH and FHA groups, and no significant differences were detected **(Supplementary Table 3).**

DISCUSSION

HH is one of the least common etiologies for female infertility and there are only a few studies on IVF characteristics in this rare condition. The key to the success of IVF is to obtain sufficient and high-quality oocytes and embryos, which is closely related to the dosage of Gn used in IVF. Generally, one patient's age and ovarian reserve function are the mainly considerations when we determine the Gn dosage of ovulation induction. However, it is unlikely that standard measures of the remaining oocyte pool (baseline FSH and LH levels or antral follicle numbers) will be of use, as they cannot be accurately interpreted in this population of women who have low Gn levels and lack cyclical menses. Besides, the age-dependent decline in ovarian response for patients with HH has not been established. In these situations, the question arises at which dose to start stimulation. In our study, 96 cycles started the ovarian stimulation with Gn dose between 150-300 IU, accounting for 80.67%. Although patients with HH have a long-term estrogen deficiency, their response to COS treatment was similar to the control women. 94.12% cycles obtained transferable embryos and only 7 cycles had no oocytes or embryos for transfer. The effect of ovulation induction was satisfactory and no severe OHSS occurred, providing a dose reference for clinical practice in the future.

Previous studies have documented that a larger amount of Gn and a longer duration of ovarian stimulation are needed in HH patients than that in tubal factor patients (12, 14), explained by the "dormant" ovaries that need to be primed before follicular response is achieved. However, the mean number of retrieved oocytes, implantation, fertilization, and pregnancy rates are not significantly different in comparison to the tubal group patients (12, 16). Ghaffari et al. found that despite a higher fertilization rate and higher number of grade A/B embryos transferred in the tubal factor group, the implantation, pregnancy, and LBRs are

TABLE 3 | Comparison of IVF related parameters between HMG and combined medication group.

Variables	HMG (n=90)	Combination group (n=29)	*P*
Hormone levels on HCG day			
E2 (pmol/L)	7114.5 (5072.5, 11454.25)	6894 (3744, 14798)	0.56
LH (mIU/mL)	0.18 (0.11, 0.57)	0.18 (0.11, 0.36)	0.65
P (nmol/L)	1.75 (1.21, 3.06)	2.25 (1.44, 3.28)	0.54
Duration of stimulation (d)	14 (13, 16)	14 (12, 15)	0.45
Total amount of Gn injected (IU)	3825 (2962.5, 4762.5)	3975 (3075, 4625)	0.96
No. of oocytes retrieved	10 (7.25, 14)	12 (7,15)	0.64
No. of fertilized embryos	8 (6, 10)	7 (5, 11)	0.47
No. of 2PN embryos	6 (5, 9)	5 (3, 9)	0.30
No. of non-2PN embryos	1 (0, 2)	2 (1, 2)	0.35
Fertilization rate (%)	0.81 (0.67, 0.93)	0.72 (0.52, 0.89)	0.16
2PN rate (%)	0.86 (0.71, 1)	0.8 (0.67, 0.94)	0.29
No. of transferable embryos	3 (2,7)	3 (2,6)	0.59

HCG, human chorionic gonadotropin; E2, estradiol; LH, luteinizing hormone; P, progesterone; Gn, gonadotropin; PN, pronuclear. The data are expressed by the median (25% quantile, 75% quantile), and the comparison between the two groups is performed by Wilcox test.

TABLE 4 | The cumulative live birth rates (CLBRs) over multiple complete IVF cycles of HH patients.

Cycle number	1st	2nd	≥3rd
No. of cycles (n)	81	27	11
No. of patients with at least one live birth (n)	35	12	2
LBR per cycle (n/N%)	43.21	44.44	18.18
Conservative CLBR (%) (95%CI)	43.21 (32.42, 54.00)	58.02 (47.28, 68.77)	60.49 (49.85, 71.14)
Optimal CLBR (%) (95%CI)	43.21 (32.42, 54.00)	68.45 (58.33, 78.57)	74.19 (64.66, 83.72)

LBR, live birth rate.

similar between HH groups and tubal factor group (13). In consistent with previous findings, our study confirmed that HH patients required a significantly longer stimulation and higher Gn doses than the control patients, but the IVF outcomes were comparable between the two groups. Furthermore, in our study, both GnRH-a and GnRH-ant regimes were evaluated while in previous studies, only a standard long protocol was discussed in the control group. In our study, HH patients were further divided into CHH and AHH subgroups, and the results indicated that although the etiology and basic treatment of HH subgroups were different, yet they have no difference in the outcomes of IVF treatment, which had not been reported in previous literature. CLBR has been suggested as a suitable way of reporting success of an IVF program which incorporates fresh as well as thawed frozen embryo transfer (17). From the patients' perspectives, CLBR is more important since it better summarizes the chance of a live birth over an entire treatment period (18, 19). One highlight of our study is that the CLBR of all cycles was analyzed, and we found that the conservative and optimal CLBRs after second oocyte retrieval cycles could reach 58.02% and 68.45%, respectively. By the fourth cycle, they reached 60.49% and 74.19%, respectively. The above data suggested that it is worthwhile to try at least two cycles of oocyte retrieval for HH patients. A recent study of CLBRs based on multicenter reproductive clinical data from the general Chinese population indicated that by the fourth transfer cycle, the conservative and optimal estimates of CLBRs are 52.95% and 77.30% in women under the age of 30 (20). Another retrospective cohort study performed among 20,687 women undergoing IVF from 2007 to 2016 found that the conservative CLBRs of the first, the second, the third, and the fourth cycles are 50.74%, 60.11%, 62.88%, and 63.75%, respectively, while the corresponding optimal CLBRs are 50.74%, 65.87%, 73.51%, and 77.61% (21). The CLBR of HH patients we reported in the present study was similar to that of non-HH patients reported in the above literature.

Due to the lack of long-term hormonal stimulation, the size of the uterus in HH patients is small. Although the uterus size is promoted with HRT before ovulation induction, concerns still arose about whether pregnancy-related risk such as premature

TABLE 5 | Comparison of clinical and cycle characteristics between congenital hypogonadotropic hypogonadism (CHH) and acquired hypogonadotropic hypogonadism (AHH) groups.

Variables	CHH (n=41)	AHH (n=40)	*P*
Age (y)	29 (27, 32)	31 (28.75, 32.5)	0.090
BMI (kg/m^2)	21.48 (19.48, 24.24)	20.97 (20.1, 23.59)	0.706
Basal serum hormonal level			
FSH (mIU/mL)	0.83 (0.28, 2.98)	1.13 (0.4, 2.55)	0.775
LH (mIU/mL)	0.27 (0.1, 1.02)	0.38 (0.1, 0.75)	0.844
E2 (pmol/L)	91.4 (73.4, 139)	73.4 (73.4, 107.5)	0.094
A (nmol/L)	4.7 (3.77, 6.2)	4.06 (2.98, 5.86)	0.322
PRL (ng/mL)	5.6 (4.58, 6.9)	6.31 (3.79, 9.91)	0.762
Hormone levels on HCG day			
E2 (pmol/L)	6575 (4302, 10984)	6960 (5611, 13117.5)	0.643
LH (mIU/mL)	0.18 (0.11, 0.58)	0.18 (0.11, 0.41)	0.434
P (nmol/L)	1.69 (1.34, 3.4)	1.84 (1.4, 2.61)	0.640
Duration of stimulation (d)	14 (13, 17)	14 (13, 15)	0.375
Total amount of Gn injected (IU)	3537.5 (3018.75, 4631.25)	3337.5 (2568.75, 4218.75)	0.204
No. of oocytes retrieved	11 (7, 12)	12 (7, 16)	0.146
No. of fertilized embryos	8 (5, 9)	9 (6, 12.25)	0.127
No. of 2PN embryos	6.5 (3, 9)	6.5 (5, 9.5)	0.252
No. of non-2PN embryos	1 (0, 2)	2 (0.75, 2)	0.358
Fertilization rate	0.77 (0.63, 0.94)	0.81 (0.66, 0.97)	0.716
2PN rate	0.85 (0.67, 1)	0.85 (0.75, 0.96)	0.547
No. of transferable embryos	3 (2, 8.25)	4 (2, 8)	0.223

BMI, body mass index; FSH, follicle stimulating hormone; LH, luteinizing hormone; E2, estradiol; A, androstenedione; PRL, prolactin; HCG, human chorionic gonadotropin; Gn, gonadotropin; PN, pronuclear. The data are expressed by the median (25% quantile, 75% quantile), and the comparison between the two groups is performed by Wilcox test.

delivery is increased in HH patients. In our study, among the 7 PTBs, only 1 preterm infant delivered at 29 weeks of gestation, and the other 6 delivered at 36 weeks. All the preterm infants had satisfactory postpartum scores and were in good health. One patient of twin pregnancy suffered abortion because of premature rupture of membranes at 26 weeks of gestation after transferring two embryos in a fresh cycle, and then delivered at term after a single blastocyst transfer in an artificial cycle. One meta-analysis of cohort studies reported that PTB occurs in 10.1% IVF/ICSI and 5.5% spontaneously conceived pregnancies (22). Another prospective follow-up study conducted by Moini et al. reported that PTB rate of twins conceived by ART before 36 weeks could be as high as 51.30% (118/230) and 36% delivered at 32-36 weeks (23). In our study, the PTB rates of singletons and twin pregnancy in HH patients were 8.33% (3/36) and 30.77% (4/13), respectively, which were lower than the reported PTB rates, indicating that pregnancy-related risk was not increased in HH patients. To our knowledge, this study was the first to report the PTB rate in HH patients. It should be noted that due to the small size of twin live births, it is necessary to accumulate data for further confirmation. As twin pregnancies are at a higher risk, singleton pregnancy is strongly recommended for HH patients.

Our study firstly assessed the efficacy of different ovulation induction drugs in patients with HH. IVF-related parameters, such as hormone levels on HCG trigger day, Gn stimulation and dosage, No. of oocytes retrieved, fertilized embryos, transferable embryos, fertilization rate, and 2PN rate, were all comparable between the two groups, indicating that there was no difference in the effects between HMG alone and combination of rFSH and HMG. Compared with rFSH, HMG is much more cost-effective and could be selected as the preferred choice. Herein, ovulation induction with HMG alone is the most commonly used protocol in our center.

Anti-Mullerian hormone (AMH) is an established marker of reproductive potential used in recent years and it does not vary considerably with physiologic fluctuations in gonadotropin levels and remains reasonably stable throughout the menstrual cycle (24). Most of women with HH may have normal or high AMH levels (25, 26), while some other studies indicated that severe and/or prolonged deficiencies in GnRH and Gn production may lower AMH concentrations (27, 28).Whether AMH levels could be used to guide fertility treatment in women with HH still need further investigation. Due to the long span of our cases, AMH was not measured at that time. Therefore, our study was unable to analyze the relationship between AMH and ovarian response. In the further research, we will discuss the characteristics of AMH in HH patients and the application value in ovulation induction program in this population.

In conclusion, we found that the results of IVF-ET in HH patients were comparable to those in women with tubal factor of oviduct factor infertility and there was no difference in IVF outcomes among different subgroups of HH. For HH patients, HMG is cost-effective for ovulation and could be considered as the first choice. Encouragingly, the PTB rate was not increased and the CLBR of HH patients were satisfactory. Therefore, IVF-ET can be successfully employed in women with HH and patients can get good pregnancy outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Peking University Third Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

YW, RL and JQ supervised the entire study and participated in the interpretation of the study data and revisions to the article. C-MZ collected the data and drafted the manuscript. RY and PL participated in the article drafting. HZ and L-XC conducted the statistical analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.850126/full#supplementary-material

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MINI REVIEW
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Bone Perspectives in Functional Hypothalamic Amenorrhoea: An Update and Future Avenues

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One of the most important and potentially long-lasting detrimental consequences of Functional Hypothalamic Amenorrhoea (FHA) is on skeletal homeostasis. Beyond oestrogen deficiency, FHA is associated with a cascade of additional neuro-endocrine and metabolic alterations, some adaptive, but which combine to disrupt skeletal homeostasis. Ultimately, this leads to a two-fold increased risk of fractures in women with FHA compared to healthy eumenorrhoeic women. Although the cornerstone of management of FHA-related bone loss remains recovery of menses *via* restoration of metabolic/psychological balance, there is rapidly developing evidence for hormonal manipulations (with a particular emphasis on route of administration) and other pharmacological treatments that can protect or improve skeletal homeostasis in FHA. In this mini-review, we provide an update on the pathophysiology, clinical management and future avenues in the field from a bone perspective.

Keywords: functional hypothalamic amenorrhoea, bone mineral density, osteoporosis, fractures, HRT, IGF1, kisspeptin

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INTRODUCTION

Functional Hypothalamic Amenorrhoea (FHA) results from the suppression of the hypothalamic control of the reproductive axis, resulting in the cessation of menses (in the absence of an organic cause). Negative energy conditions with weight loss, such as in Anorexia Nervosa (AN) or without significant weight loss (but low body fat) such as in training athletes, and psychological stress, are the main aetiologies predisposing to FHA. AN affects approximately 0.2-4% of women, with the majority experiencing amenorrhoea (1, 2). Indeed, in female athletes, the reported prevalence of secondary amenorrhoea is up to 60% (3).

Irrespective of the aetiology, FHA has detrimental effects on the skeleton through disruption of normal skeletal homeostasis, ultimately resulting in an increased risk of fractures. Therefore, it is crucial to fully appreciate the factors implicated in bone impairments in this condition. Importantly, different aetiologies of FHA and their time of onset (e.g. adolescent versus adult) are associated with characteristic neuroendocrine changes which have distinct effects on skeletal homeostasis and fracture risk (4, 5). Therefore, bone management may be tailored accordingly.

The aim of this mini-review is to give an overview of the effects of FHA on bone, specifically how they differ according to the underlying aetiology. Furthermore, we discuss current and future

treatment avenues and identify gaps in the literature to inform future research, thereby providing an update for the field.

FHA AND BONE, ACCORDING TO AETIOLOGY

A summary of the aetiologies for FHA as discussed above, and their detrimental effects on the skeleton are displayed in **Figure 1**.

Anorexia Nervosa

There is an abundance of evidence for low bone mineral density (BMD) and increased risk of fractures in FHA due to AN. In a cross-sectional study of 214 women with AN aged 17-45 years, over half had osteopenia and a third were osteoporotic. Furthermore, thirty percent of the cohort reported a previous fracture (6). This was replicated in a cohort of 60 adolescent girls with AN, where 52% had a reduced BMD (based on Z score of < -1) (7). Additionally, indices of bone quality at the microarchitectural level and bone strength, as assessed by High-Resolution peripheral Quantitative CT (HR-pQCT) were also negatively affected (8). In keeping with this high fracture risk, another study observed that the incidence rate of fracture in patients with AN (mean age 21.2 ± 9.2 years, with 94% of the cohort being female), is nearly doubled, compared to controls matched for age and gender. Furthermore, this increased risk persisted beyond 10 years from diagnosis suggesting irreversible bone impairment (9).

The issue of bone fragility is compounded by the fact that AN is predominantly a condition of younger women, typically in adolescence. This corresponds to a critical time for attaining peak bone mass (PBM). Indeed, most of the PBM is acquired before the age of 19 in females (10). In a long-term retrospective follow-up study of women who acquired AN during puberty (and therefore likely failed to achieve PBM), an increased risk of fractures was observed as far as 38 years after diagnosis, with a cumulative incidence risk of 57% at 40 years (11). In another study of over 400 participants, the lifetime prevalence of fractures was 59.8% higher in adolescents with AN, compared to healthy controls. Interestingly, this was not associated with any major reduction in axial BMD (12). A possible explanation relates to the limitation of using Dual-Energy X-ray Absorptiometry (DEXA) in this young cohort, where changes in bone microarchitecture and strength, are not adequately captured. These observations have important implications with regards to treatment strategies and the importance of early interventions to minimise long-term fracture risk.

Following on from this, it is relevant from a management perspective to note a key difference between adolescents and adults with AN, with respect to underlying bone turnover. Adolescent girls with AN, have reduced bone formation with fairly normal bone resorption, whereas adult women have reduced bone formation with markedly increased bone resorption. Overall this results in low remodelling in adolescents but higher remodelling in adult women with FHA (13). This difference suggests a limited benefit for the use of anti-resorptive agents in adolescence which has been borne out in clinical studies and highlights the different responses to treatment dependent on time of onset of FHA (14).

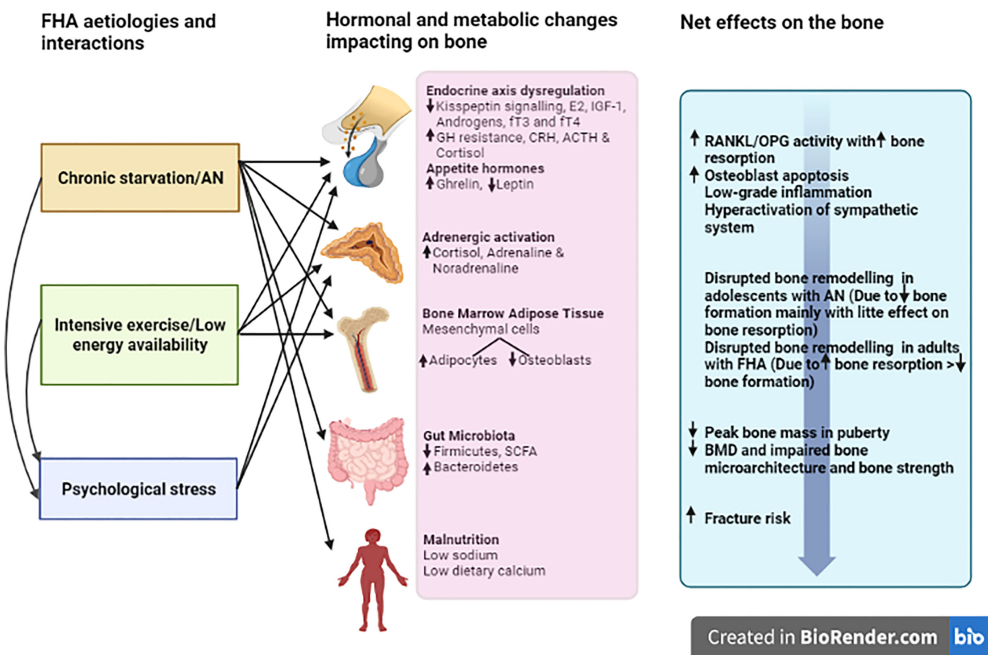

FIGURE 1 | Pathophysiology and the effects of FHA on bone.

Exercise

Exercise can be seen as a double-edged sword. In healthy populations, weight-bearing exercise has been shown to benefit BMD and a positive legacy effect is present in ex-athletes (15, 16). This was demonstrated in a study of 48 overweight adults randomised to either calorie restriction or weight-bearing exercise for a year. Despite comparable weight loss of around 8-10%, giving an approximate mean BMI of 24 kg/m^2, only the calorie-restricted group experienced a reduction in lumbar BMD (mean 2.2%), suggesting a protective effect of exercise on BMD in the face of negative energy balance (17). However, it is clear that extensive exercise coupled with low energy intake can lead to FHA and bone loss, in the so-called Female Athlete Triad. This triad consists of 3 inter-linked conditions: low energy availability, menstrual disruption and low BMD (18). Studies suggest a minimum calorie intake threshold of approximately 30 kcal/kg lean body mass/day, is required to maintain reproductive axis function (19, 20). However, this concept has been disputed by others. Lieberman et al. did not identify a specific energy threshold that induced menstrual disturbances in their cohort of women (randomised into low, moderate or high energy deficit interventional groups). However, using the threshold of 30 kcal/kg lean body mass/day in their cohort, they estimated the probability of inducing menstrual disturbances to be over 50% (21). Therefore in practice, there seems to be a spectrum of energy balance set points, at which menstrual disruption occurs at an individual level, likely related to genetic and other individual factors (22).

Regarding lifetime fracture risk, this was almost double in athletes with amenorrhoea (AA) compared to athletes with eumenorrhoea (AE) and four-fold higher compared to non-athletes (NA), in a retrospective study of 175 women. Stress fractures occurred in 32% versus 5.9% versus 0% in the AA, AE and NA cohorts respectively. Furthermore, bone microarchitecture was more negatively affected in AA, especially in those who sustained multiple stress fractures highlighting the detrimental combination of excess exercise with amenorrhoea (23).

There are some salient differences worth noting in the bone sequelae of FHA depending on an AN or exercise aetiology. In a recent study, Kandemir et al. compared bone parameters in women with AN (with or without amenorrhoea) to normal-weight athletes with oligomenorrhoea (AO) and normal-weight eumenorrhoeic controls. They observed a lower BMD and greater impairment of bone microarchitecture at all sites assessed in the AN group, compared to AO and control groups. The AO group demonstrated a lower BMD at the lumbar spine only relative to controls, and bone microarchitectural parameters were less impaired, especially at the weight-bearing tibia compared to the AN group. This highlights the greater severity of bone impairments in AN, relative to a protective bone effect during weight-bearing exercise with weight preservation, despite oligomenorrhoea. However, fracture rates were similar in AN and AO, although the latter displayed a predilection for stress fractures (which athletes are inherently more at risk of). Indeed, stress fractures were 15 times higher in the AO group compared to controls, and 7.5 times higher in the AO group compared to AN (4). Limitations of this study included its cross-sectional design and self-reporting of fractures. However, it undoubtedly highlights the different severities of bone impairment depending on underlying aetiology of amenorrhoea/oligomenorrhoea.

Psychological Stress

Psychological stress is an under-appreciated but important cause of FHA. Psychological stress can independently suppress the reproductive axis but commonly co-exists and interacts synergistically with other stressors such as energy restriction and over-exercising (as above), resulting in FHA. In a recent study involving 61 exercising women by Strock et al, women with amenorrhoea showed a greater drive for thinness and a greater need for social approval than women with eumenorrhoea. Furthermore, this was positively associated with indicators of psychological stress and depression, assessed by questionnaires. This was despite both groups having comparable exercise intensity and energy intake, thus highlighting the role of stress in FHA (24). Others have also reported that women with FHA have more dysfunctional attitudes (such as drive for perfectionism, rigidity of ideas, preoccupation of being judged), more depressive symptoms and are less able to cope with stressors than eumenorrhoeic controls. These specific personality traits of women with FHA, therefore make them more susceptible to life stressors (25, 26).

These studies demonstrate an association of psychological stress with FHA but do not identify causality. However, psychological stress is a key activator of the Hypothalmic-Pituitary-Adrenal (HPA) axis, promoting cortisol secretion, which in excess has established negative effects on skeletal homeostasis (and reproductive function). However, there exist additional mechanisms linking psychological stress with bone disruption. Low grade inflammation as evidenced by increased pro-inflammatory markers (such as tumour necrosis alpha-α), has been associated with acute stress and shown to cause upregulation of Receptor activator nuclear factor kappa-B ligand (RANKL) signalling and therefore increased bone resorption in pre-clinical studies (27, 28). Stress-induced hyperactivation of the sympathetic system has also been proposed as another mechanism. Indeed, receptors for noradrenaline are present on osteoclasts and osteoblasts (29) and stress-induced bone loss is observed in the context of elevated noradrenaline levels in mice, while propranolol, a β-adrenergic antagonist, blocks this negative effect (30). Taken together, there is not only evidence that psychological stress can cause FHA as well as associate with AN/exercise, but that psychological stress itself can directly impair skeletal homeostasis.

ENDOCRINE MEDIATORS OF BONE LOSS IN FHA

The key defects in FHA are attenuated hypothalamic secretion of kisspeptin and downstream gonadotropin-releasing hormone (GnRH) (31). This results in inadequate secretion of downstream follicle-stimulating hormone and luteinising hormone to sustain normal menstrual cyclicity. The negative energy balance, low body fat and/or psychological stress result in

the disruption of multiple neuro-endocrine signals (**Figure 1**) leading to failure of the downstream reproductive axis culminating in oestrogen deficiency and detrimental effects on skeletal homeostasis (32).

Reduced Kisspeptin

Kisspeptin (secreted by kisspeptin neurons) is the master hypothalamic regulator of the reproductive axis and controls downstream GnRH secretion through kisspeptin receptors located upon GnRH neurons (33). In FHA, kisspeptin secretion has recently been shown to be reduced (34), while conversely administration of kisspeptin to patients with FHA can restore downstream pulsatile LH secretion (35). Crucially, kisspeptin neurons receive multiple neuro-endocrine and metabolic signals that can be disrupted in FHA, and so serve to orchestrate the downstream reproductive axis based on these inputs. Although kisspeptin secretion regulates downstream classical reproductive hormones crucial to skeletal homeostasis (predominantly oestrogen and testosterone), recent data has identified direct positive effects for kisspeptin in bone (36–39). However, although in FHA there is reduced kisspeptin signalling in the hypothalamus, it is currently unknown if kisspeptin signalling is also reduced in bone.

Reduced Oestrogen

Oestrogen receptors are present on the three main bone cells: osteoclasts, osteoblasts and osteocytes. Oestrogen inhibits bone resorption directly by inducing osteoclastic apoptosis and indirectly by disrupting the RANKL/Osteoprotegerin (OPG) pathway. Recent work suggests that RANKL expression on bone lining cells (derived from osteoblasts) is a key mediator of oestrogen-controlled bone resorption (40). In addition, further new data has identified oestrogen-induced secretion of semaphorin 3A, a protein known to reduce bone resorption and increase bone formation, from osteocytes (41), as well as anti-apoptotic effects by oestrogen on osteoblasts (via promotion of autophagy) (42). Taken together, the net effect of oestrogen is a reduction in bone remodelling (due to greater effect on reducing bone resorption compared to increasing bone formation). Therefore, oestrogen deficiency states are characterised by increased bone remodelling resulting in disrupted skeletal homeostasis. In the early menopause transition, BMD decreases by about 2% per year (43). Further demonstrating the impact of oestrogen deficiency, eumenorrhoeic women with AN have higher BMD than amenorrhoeic (i.e. lower oestrogen levels) women with AN, although both groups display lower than normal BMD (T score -1.2 in eumenorrhoeic versus -2.3 in amenorrhoeic women) (44). This highlights the dominating detrimental impact of oestrogen deficiency as seen in FHA beyond other nutritional and endocrine effects of anorexia nervosa.

Reduced Androgens

Low levels of testosterone and DHEA are observed in AN (45) with associated impairments in bone microarchitecture (46). However conflicting findings of high or normal levels of androgens have been observed in athletes and normal-weight women with FHA (45–47). Although androgens mediate most of their effect on bone indirectly from aromatisation into oestrogens, androgens themselves are also important in women predominantly for trabecular bone (48).

Reduced Leptin

Leptin is reduced in FHA mainly secondary to acute calorie restriction and stress, independent of weight loss (49, 50). Leptin has both central and peripheral actions on bone. Centrally, low leptin levels reduce the secretion of Insulin Growth Factor-1 (IGF-1), oestrogen and thyroid hormones, which all normally have positive bone effects (51). These hormonal reductions are part of a necessary adaptive energy-sparing response, to minimise growth, reproduction and metabolism respectively. Peripherally, leptin receptors are present on osteoblasts with possible anabolic roles in bones by enhancing osteoblast proliferation (52, 53). Furthermore, *in vitro* studies suggest a role for leptin-driven differentiation of human marrow stem cells into osteoblasts further supporting an anabolic role (54).

Elevated Ghrelin

In contrast to leptin, ghrelin levels are elevated in women with FHA (55). This response is presumed to be physiological to stimulate calorie intake and restore energy balance. Ghrelin is also a known growth hormone (GH) secretagogue and may contribute to excess GH secretion in AN (55). Interestingly, elevated ghrelin levels have been associated with a delayed return to menstrual cyclicity in women with persistent disordered eating in FHA, despite normalisation of weight and leptin levels. This suggests a direct effect of ghrelin on the reproductive axis in FHA (56). From a bone perspective, ghrelin directly stimulates osteoblast proliferation *in vitro*, and increases BMD in rodents *in vivo* (57). A similar anabolic effect on bone has also been observed following intracerebroventricular administration of ghrelin to rodents, independent of body weight (58). Taken together, these studies suggest central and peripheral positive effects of ghrelin on bone. However, although in FHA, ghrelin levels may be raised, this beneficial effect is far outweighed by the repercussions of other hormonal changes such as hypoestrogenism on bones.

Elevated GH and Reduced IGF-1

IGF-1 levels are reduced by up to 50% in AN despite increased GH, in keeping with a state of GH resistance (59, 60). IGF-1 has established anabolic effects on bone through increases in osteoblast activity and collagen synthesis (61). Crucially, IGF-1 has a key role in the gain of bone mass during puberty and correlates positively with BMD and bone formation markers in adolescent girls with AN and with bone microarchitecture in adult women with AN (62, 63). This further highlights the potential longer-term detrimental effects of FHA on bone when there is failure to achieve an optimal PBM in younger years.

Elevated Cortisol

Increased levels of Corticotrophin-Releasing Hormone (CRH), corticotrophin (ACTH) and downstream 24-hour cortisol levels are a consistent feature of FHA (32, 64). This is due to physical or

psychological stress activating the HPA axis with the increases in cortisol capable of further suppressing the reproductive axis (32).

Hypercortisolaemia itself can contribute to bone loss. In a study of normal-weight and AN-induced adult women with FHA, hypercortisolaemia was observed in both groups, and was negatively correlated with BMD (65). There are multiple mechanisms for the detrimental effects of glucocorticoids (such as cortisol) on bone beyond the scope of this mini-review but include reduced gut absorption and increased renal loss of calcium, as well as increased osteoblast apoptosis and enhanced bone resorption *via* the RANKL/OPG pathway (66).

Reduced Thyroid Hormones

AN is associated with reduced levels of free T3 (fT3) and free T4 (fT4) compared to controls, similar to the nonthyroidal illness syndrome observed in patients with systemic illness (67). Similarly, lower thyroidal hormonal levels have been reported in FHA due to exercise, compared to their eumenorrhoeic counterparts. In this study, reduced T3 and T4 levels were associated with a prolonged post-exercise muscle recovery rate, as assessed by phosphate recovery kinetics (68). In a more recent study involving women with FHA (but not AN), those with fT3 levels below the normal range had a lower BMD at the spine and hip as well as lower circulating osteocalcin levels (a marker of osteoblastic activity), compared to those with preserved fT3 levels (mean lumbar T score range: -0.6 to -3.4 versus 0.2 to -2.9 respectively; mean hip T score range: -0.4 to -2 versus 1.8 to -1.6 respectively). A compensatory increase in oxidative stress, driven by low fT3 levels, has been proposed as the underlying mechanism impairing skeletal homeostasis (69).

Increased Bone Marrow Adipose Tissue

Bone marrow adipose tissue (BMAT) is increased in energy deficient states (such as AN and exercise-induced FHA) due to preferential differentiation of mesenchymal stem cells to adipocytes (at the expense of osteoblasts) and this increase correlates inversely with BMD (70) (71). *In vitro* studies demonstrate that bone marrow adipocytes release inflammatory cytokines and RANKL, which promote osteoclastogenesis, while the secretion of saturated fatty acids can also disrupt osteoblast function and lifespan (72–74). Putative mediators of the increase in BMAT include IGF-1, leptin, oestrogens, and pre-adipocyte factor-1 (75). Interestingly, a recent exploratory study in 16 women with FHA revealed that the expected increase in BMAT in this condition can be attenuated by transdermal 17β-estradiol treatment (71). Further studies in this respect and with control groups will be of great interest.

Low Sodium

Lower circulating sodium levels are a frequent feature of AN (with or without amenorrhoea). In a large cross-sectional study of over 400 women with AN, a lower sodium level (<140mmol/L) was associated with a lower BMD at both the spine and hip compared to those with a sodium level >140mmol/L (reference range: 135-145mmol/L) (76). Overt hyponatraemia is also a recognised risk factor for bone loss, osteoporosis and fractures (77). Bone loss in hyponatraemia has been attributed to mobilisation of sodium stores from the bone *via* increased bone resorption (in an attempt to correct the low sodium), inappropriate vasopressin secretion and a direct effect of hyponatraemia on osteoclast activity (78).

In summary, patients with FHA have a multitude of endocrine abnormalities (beyond oestrogen deficiency) that can contribute to the disruption of skeletal homeostasis, as illustrated in **Figure 1**.

TREATMENT

Weight Gain, Restoration of Energy Balance, Reduction in Psychological Stress

Weight gain, restoration of energy balance and reduction in psychological stress leading to restoration of menstrual cycles are the most effective management strategies for FHA-related bone loss (79). In a study by Miller et al. involving 75 women with AN, weight gain especially lean body mass and resumption of menstrual function were both necessary for BMD recovery at the spine and hips (80). In contrast, improvement in BMD with weight restoration but without restoration of menses has been observed (14, 81), while others did not observe any change in BMD following weight gain alone (82, 83). These latter discrepant findings may be due to limited numbers, lack of controls, non-randomised study design and limited follow-up time, which may be insufficient to capture changes in BMD. However, it is worth noting that even if no incremental effect of weight gain was reported on BMD in some studies, a deterioration over time was nevertheless not observed, which is in itself a positive outcome (82, 83).

Unfortunately, achieving and maintaining a positive energy balance long-term is challenging for most women with FHA. Indeed, only about 60% of women with AN achieve recovery at 22 years (84). Additionally, AN is associated with a long-term increased risk of fractures in later life, irrespective of recovery (9). Even in athlete-related amenorrhoea, non-pharmacological intervention (increased dietary intake and/or decreased exercise) led to return of menses in only 17.6% of college athletes, while in the recent randomised controlled 'REFUEL' study, an increase in energy intake of about 330 kcal/day in exercising women with oligo/amenorrhea improved menstrual function in only 64% at 1 year (85, 86). Hence, there is a compelling need for effective long-term pharmacological replacement/treatment for women with FHA to protect their bones as the aforementioned non-pharmacological methods are challenging and not always fully effective.

Oestrogen Treatment

Oestrogen replacement/treatment studies in FHA have revealed notable bone results related to the route, formulation and dosage of oestrogen. An up-to-date summary of clinical trials and other key studies related to bone treatment are reported in **Table 1**.

In a recent pivotal study, 121 oligo-amenorrhoeic athletes, aged 14-25 years, were randomised to a transdermal patch providing a

'physiological' 100 mcg 17β-estradiol, a combined oral contraceptive pill (COCP, containing a 'supraphysiological' 30 μg Ethinyl-Estradiol (EE)) or no oestrogen. Only the transdermal patch group exhibited BMD improvements at 12 months (approximately 3% at the lumbar spine and 5% at the femoral neck). Surprisingly, those on the COCP had a (nonsignificant) trend to a worse BMD compared to controls mainly at the total hip (97). Crucially, there were no significant differences in weight or menstrual function change between the patch and pill groups by the end of the study, that could have confounded these results. Microarchitectural indices also improved significantly in the patch versus COCP group, especially at the tibia (107). These aforementioned findings in oligo-amenorrhoeic athletes are mirrored in females with AN. Misra et al. showed that 18 months of transdermal 17β-estradiol (100 mcg patch twice weekly) but not the COCP (35 μg of EE + 0.18-0.28 mg of norgestimate) led to an improvement of 2.6% in lumbar BMD in adolescents with AN (96). In a separate group of adolescents with AN, treatment with a triphasic COCP (35 μg of EE + 0.18–0.25 mg of norgestimate) for 13 months, did not lead to any significant change in lumbar or hip BMD (94). Similarly, in a recent 6-month pilot study, Resulaj et al. observed an increase of 2% in the lumbar BMD of women with AN (mean age 37 years), following transdermal oestradiol (45 mcg/day), although there was no control group (98). In contrast to transdermal physiological dose oestrogen, the COCP has not shown any convincing benefits (in terms of BMD) in adult women with FHA due to AN or exercise (90, 95).

These differing actions of oestrogen treatment have been mainly attributed to the route of its administration. Oral COCP inhibits IGF-1 production *via* first-pass hepatic metabolism, from which transdermal oestrogen is exempt. Indeed, a reduction in IGF-1 levels, associated with a greater fall in P1NP (a marker of osteoblastic activity) levels is observed during COCP treatment, but not with transdermal 17β-estradiol. Although the oestrogen dose is higher in studies of the COCP compared to transdermal oestrogen, even lower oral doses of oestrogen (1mg 17β-oestradiol) have suppressive effects on IGF-1 compared to transdermal oestrogen (108). Furthermore, oral oestrogens can increase hepatic sex hormone binding globulin levels, thereby reducing bioavailable oestrogen to the detriment of skeletal homeostasis (109).

In summary, the body of evidence for the positive effect of oestrogen treatment on bone in FHA defines a beneficial effect for transdermal oestrogen replacement over the COCP, with promising recent results (96, 97). This concept was confirmed in a very recent meta-analysis of the effects of oral contraceptives, conjugated oestrogens and transdermal oestrogens in FHA, with the latter showing consistent superiority in terms of BMD gains (110). However, it is worth noting inherent difficulties in these studies, with small numbers, high drop-out rates, relatively short follow-up, heterogeneity in types and doses of oestrogen (and progestin) used, and crucially the lack of fracture-related data. Therefore, further work is warranted to assess the doses (physiological (i.e. replacement) versus supraphysiological), the types (17β-estradiol versus ethinyl-oestradiol versus conjugated oestrogens) and the routes of administration of oestrogen (transdermal versus oral) to clearly define the optimal treatment strategy. Currently, the data point to transdermal oestrogen replacement as the optimal strategy.

Androgen Treatment

Transdermal testosterone replacement and DHEA do not increase BMD in women with AN (with and without amenorrhoea) at 12 months (**Table 1**) (99, 101). However, a combination of DHEA and COCP led to stabilisation of BMD over 18 months, relative to placebo, where a drop in BMD was observed (100). Further studies are required to clarify the independent benefits of androgen treatment.

IGF1 Treatment

Given the aforementioned suppression of IGF1 observed in FHA, it is not surprising that recombinant human IGF-1 (in combination with a COCP), led to an increase in lumbar BMD compared to placebo, by 1.8% versus -1% respectively at 9 months in women with AN and osteoporosis (aged between 18-38 years). The corresponding changes in lumbar BMD with IGF-1 or COCP monotherapy were 0.3% and -0.2% respectively (See **Table 1**). Longer studies of IGF-1 treatment are warranted given that the duration was only 9 months (90).

Leptin Treatment

Leptin treatment has also been the subject of study in FHA. Subcutaneous leptin administration can restore reproductive axis function with return of menses in a third of women with FHA (due to AN) with associated reductions in cortisol, and increases in IGF-1, thyroid hormones and bone formation markers (102). A 2-year study with daily subcutaneous metreleptin injection, culminated in 4-6% gain in BMD at the lumbar spine in exercising women (103). However, leptin treatment was associated with approximately 3% weight loss which has ultimately restricted its development for FHA despite these promising biochemical and bone outcomes. See **Table 1** for a summary of studies investigating leptin treatment in FHA.

Bisphosphonates and Denosumab

There is a limited number of studies evaluating the benefits of bisphosphonates in FHA-related bone loss. These are small prospective studies looking at alendronate, risedronate and etidonate (14, 101, 111). Only risedronate showed a significant increase in BMD at the spine and hip by approximately 4% and 2% respectively, at 9-12 months in women with AN, most of whom were not experiencing endogenous menses (101, 104). However, no positive effect of bisphosphonates has been observed in adolescents with AN (14); presumably due to reduced underlying bone turnover as discussed previously. Key points of these studies are outlined in **Table 1**.

There are case reports supporting the use of denosumab in osteoporotic women with AN (aged 37-42 years, BMI 12.2-18.3 kg/m^2) although menstrual status was not reported (112). However, no clinical trials have investigated denosumab in FHA to-date.

The barriers to using bisphosphonates in FHA are their prolonged half-lives with a small but potential teratogenic

TABLE 1 | Up-to-date summary of oestrogen treatment studies and other hormonal/pharmacological interventional studies in women with FHA.

	Study	Study Design	Subjects	Aetiology of FHA	Age(years)	Duration (months)	Intervention	Change in BMD
				Oestrogen Treatment				
1	Hergenroeder et al, 1997 (87)	RCT	24	Mixed (AN/ Athletes and Ballet dancers)	14-28	12	COCP (35μg EE + 0.5- 1.0mg norethindrone) vs 10mg medroxyprogeterone (MP) for 10 days vs placebo	• Change in lumbar BMD: 5.4% (COCP) vs -10.2% (MP) vs -0.7% (placebo). This increase in BMD with COCP was significant compared to MP and placebo. • Change in Femoral Neck BMD: 2.2% (COCP) vs -5.6% (MP) vs -2.7% (placebo). Not significantly different.
2	Gibson et al, 1999 (88)	RCT	34	Exercise (Runners)	18-34	12	Trisequens oral HRT (estriol 1mg + estradiol 2mg for 12 days, estriol 1mg + estradiol 2mg + norethisterone 1mg for 10 days, estriol 0.5mg + estradiol 1mg for 6 days) vs placebo	• Change in lumbar BMD: 4.1% (in those who became eumenorrhoeic on HRT). • Change in hip BMD: 3.8% (in those who became eumenorrheoic on HRT). • Mean change in lumbar BMD relative to placebo: 1.5% (effect reflects return of menses in placebo + withdrawals from treatment group).
3	Castelo-Blanco et al, 2001 (89)	RCT	64	Not specified	Mean 24.4	12	COCP (30μg of EE + 0.15mg desogestrel) vs COCP (15μg of EE + 0.15mg desogestrel) vs placebo	• Change in lumbar BMD: non-significant increase of 2.4% and 2.5% (COCP 30 μg and 15 μg cohorts respectively) vs -1.2% (placebo)
4	Grinspoon et al, 2002 (90)	RCT	66	AN	18-38	9	COCP (35μg EE + 0.4mg norethindrone) and recombinant human IGF-1 (rhIGF-1) or rhIGF-1 alone or COCP alone or placebo	• Changes in lumbar BMD: 1.8% (COCP + rhIGF-1), vs 0.3% (rhIGF-1) vs -0.2% (COCP) vs -1.0% (placebo). Increase in BMD with COCP + rhIGF-1 was significantly higher relative to placebo only.
5	Warren et al, 2003 (91)	RCT	24	Exercise (ballet dancers)	Mean 20.8	24	Oral conjugated oestrogen (CE), Premarin (0.625mg) for 25 days with Provera 10mg for 10 days vs placebo and vs controls (ballet dancers with normal menses)	• Changes in lumbar BMD: 5.6% (CE) vs 4.5% (placebo) vs 6.7% (controls). Not significantly different.
6	Rickenlund et al, 2004 (92)	Prospective-placebo controlled	26	Athletes (Endurance sports)	16-35	10	COCP (30μg EE + 150μg levonorgestrel) vs placebo	• Small significant increase in total body BMD with COCP (but significant weight gain among subjects during study). • No change in lumbar BMD with COCP
7	Warren et al, 2005 (93)	Open-labelled single arm extension study.	45	Not specified (but AN excluded)	18-40	10	COCP (35μg EE + 180-250 μg norgestimate)	• Change in lumbar BMD: 1.5% (COCP). Significantly higher relative to baseline.
8	Strokosch et al., 2006 (94)	RCT	112	AN	11-17	13	COCP (35μg of EE + 0.18-0.28mg of norgestimate) or placebo	• Change in lumbar BMD: 3.1% (COCP) vs 2.4% (placebo). Not significantly different. • Change in hip BMD: 1.5% (COCP) vs 1.8% (placebo). Not significantly different.
9	Cobb et al, 2007 (95)	RCT	150	Exercise (runners)	18-26	24	COCP (35μg EE + 0.3mg norgestrel) or control (no intervention given)	• Change in lumbar BMD: 1% per year (COCP, who remain amenorrhoeic). This increase in BMD was comparable to those who spontaneously regain menses but higher than those who did not in the control group.

(Continued)

TABLE 1 | Continued

	Study	Study Design	Subjects	Aetiology of FHA	Age(years)	Duration (months)	Intervention	Change in BMD
10	Misra et al, 2011 (96)	RCT	110	AN	12-18	18	Transdermal 100mcg 17β-estradiol (TE) + medroxyprogesterone 2.5mg for 10 days vs placebo	• Change in lumbar BMD: 2.6% (TE) vs 0.3% (placebo). This difference was significant. • Change in BMD at hip: 0.004% (TE) vs -1.2% (placebo). This difference was significant.
11	Ackerman et al, 2019 (97)	RCT	121	Exercise	14-25	12	TE (100mcg 17β-estradiol + micronized progesterone 200mg) vs COCP (35μg EE + 0.15mg desogestrel) vs placebo	• Change in lumbar BMD: 2.75% (TE) vs 0.3% (COCP, estimated from Graph) vs no change (placebo). • Change in neck of hip BMD: 5.25% (TE) vs 1.8% (COCP, estimated from graph) vs 2% (placebo, estimated from graph).
12	Resulaj et al, 2020 (98)	Single-arm prospective	11	AN	Mean 37.2	6	TE (45mcg/day 17β-estradiol + levonorgestrel 0.015mg)	• Significant increase in lumbar BMD by 2%.
					Androgen Treatment			
1	Gordon et al, 2002 (99)	RCT	61	AN	14-28	12	DHEAS 50mg/day vs COCP 20μg EE + 0.1mg levonorgestrel vs placebo	• Change in hip BMD: 1.7% (DHEAS and COCP). This was not significant when controlled for weight gain. • No change in lumbar BMD.
2	Divasta et al, 2012 (100)	RCT	94	AN	13-27	18	DHEAS 50mg/day with COCP 20μg EE + 0.1mg levonorgestrel vs placebo	• No change in lumbar or hip BMD in DHEAS with COCP or placebo groups.
3	Miller et al, 2011 (101)	RCT	77	AN	Mean: 25.3 (Risedronate), 27.1 (Testosterone), 25.2 (Combined), 26.9 (double-placebo)	12	Risedronate 35mg weekly vs testosterone 150μg daily patch vs risedronate 35mg weekly + testosterone 150μg daily vs placebo	• No significant change in lumber and hip BMD with testosterone. • Significant increase in lumbar BMD by 4% in risedronate group only, compared to placebo. • Significant increase in hip BMD by 2% in risedronate group only, compared to placebo.
					Leptin Treatment			
1	Welt et al, 2004 (102)	Prospective-placebo controlled	8	Exercise	19-33	3	r-metHuLeptin (0.08mg/kg) s.c daily vs placebo	• No change in total body BMD.
2	Sienkiewicz, et al. (103)	RCT	20	Exercise	18-35	Up to 24	Metreleptin (0.08-0.12mg/kg/day) vs placebo	• Significant increase in lumbar BMD by 4-6% from baseline. • No change in hip BMD.
					Denosumab Treatment			
1	Isobe et al	Retrospective case-series	3	AN	36-42	24	Denosumab 60 mg	• Changes in lumbar BMD: 15.7% (case 1), 18.6% (case 2) and N/A (case 3) relative to baseline • Changes in hip BMD: 36.1% (case 1), 11.6% (case 2), 10.7% (case 3) relative to baseline
					Bisphosphonate Treatment			
1	Golden et al, 2005 (14)	RCT	32	AN	12-21	12	Alendronate 10mg daily vs placebo	• Changes in lumbar spine: 3.5% (alendronate) vs 2.2% (placebo). Not significantly different. • Change in Femoral neck BMD: 4.4% (alendronate) vs 2.3% (placebo). Not significantly different.
2	Miller et al, 2011 (101)	RCT	77	AN	Mean: 25.3 (risedronate), 27.1 (testosterone), 25.2 (combined),	12	Risedronate 35mg weekly vs testosterone 150 μg daily patch vs risedronate 35 mg weekly + Testosterone 150μg daily vs placebo	• Significant increase in lumbar BMD by 4% in risedronate group only, compared to placebo. • Significant increase in hip BMD by 2% in risedronate group only, compared to placebo.

(Continued)

TABLE 1 | Continued

	Study	Study Design	Subjects	Aetiology of FHA	Age(years)	Duration (months)	Intervention	Change in BMD
					26.9 (double-placebo)			• No significant change in lumbar and hip BMD with testosterone.
3	Miller et al, 2004 (104)	Prospective-placebo controlled	10	AN	Mean: 28.6 (risedronate), 26.9 (placebo)	9	5mg risedronate daily vs placebo	• Significant increase in lumbar BMD by 4.9%, compared to placebo.
					Teriparatide Treatment			
1	Fazeli et al, 2014 (105)	RCT	21	AN	Mean 47	6	Teriparatide (20μg SC daily) or placebo	• Significant increase in lumbar BMD by 10.5% compared to placebo. • No significant changes in BMD at hip.
2	Milos et al, 2020 (106)	Prospective single-arm	10	AN	21-33	24	Teriparatide (20μg SC daily)	• Significant increase in lumbar BMD by 13.5%. • Significant increase in femoral neck BMD by 5.0%.

RCT, Randomised Clinical Trial; EE, Ethinyl Estradiol; TE, Transdermal Oestrogen; vs, versus; SC, Subcutaneously; N/A, Not Available.

(observed in rodent studies but not consistently in humans) or neonatal complication risk, in a patient population often in their reproductive years (113). Similarly, denosumab is associated with complications if used in pregnancy (114).

Teriparatide

Anabolic agents such as teriparatide have been trialled with good effect (See **Table 1**). In a randomised controlled trial (RCT) of 21 osteoporotic women with AN and a mean BMI of 17.6 kg/m^2, teriparatide resulted in a significant increase in lumbar BMD of 6% at only 6 months (105). More recently, Milos et al. provided further supporting evidence by studying a slightly younger cohort of women with AN (mean BMI 15.6 kg/m^2) with or without previous fragility fractures. Teriparatide treatment for 24 months resulted in a significant increase in BMD of 13.5% at the lumbar spine and 5% at the hip. Notably, this was independent of gain in body weight and body fat (106). However, it is worth noting that this study lacked a control group and changes in menstrual function, which may have confounded the results, were not reported. Barriers to the use of teriparatide are its limited use of up to 2 years (which may lead the clinician to reserve teriparatide for when the patient with FHA is older or use it for shorter periods at different ages), cost and the inconvenience of daily injections.

FUTURE AVENUES

Romosozumab

Future pharmaceutical avenues include the humanised monoclonal antibody to sclerostin, Romosozumab, which is approved for the treatment of post-menopausal osteoporosis. Of note sclerostin levels have been reported as unaltered or raised in adolescent and young women with AN compared to healthy controls (115, 116). Data in FHA are awaited but this suggests that women with FHA (at least due to AN) may be susceptible to sclerostin pathway inhibition. Studies are therefore warranted in this regard although safety in women of reproductive age will again need to be clearly ascertained (there are no human pregnancy data as yet).

Kisspeptin

Another recent promising avenue is kisspeptin treatment. It has previously been demonstrated that kisspeptin administration can restore LH pulsatility in women with FHA acutely while twice weekly injections for 8 weeks can stimulate the secretion of reproductive hormones without significant desensitisation (117, 118). Recent data has now emerged from a bone perspective suggesting that kisspeptin administration also can have direct positive effects in human bones. In this study we showed that kisspeptin potently stimulated osteogenic differentiation of osteoblast progenitors and inhibited bone resorption *in vitro* (by up to 53.4%), in a dose-dependent manner. Furthermore, acute kisspeptin administration to healthy young men increased osteoblast activity *in vivo*. Further studies are warranted but collectively these data suggest that kisspeptin administration could benefit skeletal homeostasis in FHA by restoring reproductive hormone secretion as well as by direct effects on bone.

Gut Microbiota

Another emerging avenue is the association of the gut microbiota with abnormal body weight. Signature changes recently reported in women with AN include a relative reduction in firmicutes and short-chain fatty acids (SCFA), and an increase in bacteroidetes, Methanobrevibacter smithii and Escherichia coli (E.coli) species (119). Some of these changes may be adaptive but a positive association between E.coli and appetite suppression at the level of the MC4 receptors has been described in rodents (120). Yan et al. demonstrated that treatment with broad spectrum antibiotic for 2 months led to depletion of the microbiota in female germ-free mice with subsequent reduction in SCFA and IGF-1 levels. In contrast, SCFA supplementation in antibiotic-treated mice for 6 weeks, restored levels of IGF-1 and improved bone mass to reflect that of non-antibiotic-treated mice (121). Further clinical studies, specifically

exploring the role of SCFA and pro and pre-biotics as potential treatment agents for bone health in FHA are now warranted.

CONCLUSION

Low BMD with an increased risk of fractures is a major complication of FHA due to a multitude of factors as updated above. Given the undoubted severity of the negative effects on bones, there remains an unmet need to clearly determine the optimal oestrogen replacement strategy as well as testing alternative and new pharmacological interventions to treat FHA-related bone loss. Current evidence favours transdermal 17β-estradiol as being the most promising intervention from an oestrogen replacement perspective, although larger and longer studies are needed to verify its long-term benefits, especially on the ultimate outcome of fractures. In addition, the potential use of romosozumab, kisspeptin and pro/prebiotics, warrant further exploration.

AUTHOR CONTRIBUTIONS

PB drafted the manuscript. AC reviewed and amended the manuscript. All authors contributed to the article and approved the submitted version.

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Luteinizing hormone supplementation in women with hypogonadotropic hypogonadism seeking fertility care: Insights from a narrative review

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The management of infertile women affected by hypogonadotropic hypogonadism (HH) or conditions mimicking it is particularly challenging. In the present narrative review, we aimed to synthesize the available evidence on the benefit (if any) of exogenous luteinizing hormone (LH) supplementation in this group of patients. Available data support LH supplementation in women with organic or functional HH. On the contrary, the benefit of exogenous LH on reproductive outcomes both in advanced maternal age patients and in cases of depletion of FSH and LH levels induced by GnRH analogues has not been demonstrated. unfortunately, the inhomogeneous study populations as well as the methodological heterogeneity between studies focused on women affected by conditions mimicking HH do not allow reliable conclusions to be drawn.

KEYWORDS

LH, LH supplementation, ART, hypogonadotropic hypogonadism, infertility

Abbreviations: ART, assisted reproductive technology; CI, Confidence interval; E2, Estradiol; FSH, Follicle stimulating hormone; FSHR, FSH receptor; GnRH, Gonadotropin releasing hormone; hCG, human chorionic gonadotropin; HH, Hypogonadotropic hypogonadism; hMG, human menopausal gonadotropin; hMG-HP, highly purified hMG; ICMART, International Committee for Monitoring Assisted Reproductive Technologies; IVF, *in vitro* fertilization; LH, Luteinizing hormone; LHCGR, LH/HCG receptor; OCP, oral contraceptive pill; OR, odds ratio; OS, Ovarian stimulation; RCT, randomized clinical trial; rFSH, recombinant FSH; rLH, recombinant LH; WHO, World Health Organization.

Introduction

According to the International Committee for Monitoring Assisted Reproductive Technologies (ICMART), hypogonadotropic hypogonadism (HH) is defined as "gonadal failure associated with reduced gametogenesis and reduced gonadal steroid production due to reduced gonadotropin production or action" (1). The possible causes of HH are reported in Table 1 (2–4). The ICMART definition follows the traditional concept but, at the same time, broadens its boundaries by including an exclusively functional etiopathogenesis (2).

Women affected by the conditions listed in Table 1 are, in the vast majority of cases, infertile and, therefore, they frequently refer to an Infertility Unit to receive a diagnosis and a proper treatment. Women selected for assisted reproductive technology (ART) are at increased risk of conditions mimicking HH for at least three other reasons: i) in most cases, they approach treatment at an advanced stage of their reproductive life span; ii) ovarian stimulation (OS) protocols include gonadotropin releasing hormone (GnRH) analogues; iii) a low affinity of luteinizing hormone (LH) and follicle stimulating hormone (FSH) to their receptors are often unrecognized till an unexpected low response to OS for ART (5–8).

The management of patients affected by organic, functional or iatrogenic HH seeking fertility care is particularly challenging and several therapeutic strategies have been proposed.

In the present narrative review, we focused on exogenous LH supplementation. LH exerts two crucial activities during folliculogenesis. First of all, it induces androgen production in theca cells. Second, during the intermediate follicular phase, it cooperates with FSH in stimulating the local production of inhibin B and growth factors. Among these, insulin like growth factors 1 and 2, which are expressed in both granulosa and theca cells, are of utmost importance in promoting follicular maturation (9–11). Based on the notions learned from physiology, the administration of LH combined with FSH during OS in LH deficient women was hypothesized to have beneficial effects on growing follicles and, as a result, on the fertility treatments success rate (9, 10). Furthermore, the addition of exogenous LH might benefit the endometrium by decreasing the risk of a premature progesterone rise (12).

Herein, we aimed at evaluating the effect (if any) of exogenous LH supplementation on fertility related outcomes in women suffering from HH.

Materials and methods

The present narrative review was restricted to published research articles that reported data relevant to the effect of LH supplementation in women affected by HH or conditions mimicking HH,on fertility treatments outcomes. We searched MEDLINE, Embase and Scopus, from database inception to 1 May 2022. Searches were limited to studies in humans and were conducted using the following terms: 'hypogonadotropic hypogonadism' and 'luteinizing hormone supplementation' OR 'luteinizing hormone supplementation' and 'ovulation induction' OR 'luteinizing hormone' and '*in vitro* fertilization' OR 'luteinizing hormone' and 'intracitoplasmic sperm injection' OR 'luteinizing hormone' and ' advanced maternal age' OR 'luteinizing hormone' and 'GnRH analogue' OR 'luteinizing hormone supplementation' and 'gonadotropin's receptor'.

Women with depleted basal FSH and LH serum levels

Organic functional HH (World Health Organization (WHO) group I anovulation) determines anovulation,

TABLE 1 Causes of hypogonadotropic hypogonadism in women.

Etiology
A. Congenital HH
a.1 Kallmann Syndrome, Prader Willy Syndrome or other genetic mutation
a.2 Idiopathic hypogonadotropic hypogonadism
B. Acquired HH
b.1 ORGANIC
b.1.1. Infiltrative or infectious pituitary lesions (e.g., tumor, hemochromatosis, sarcoidosis, histiocytosis X, thalassemia, granuloma, abscess)
b.2 FUNCTIONAL
b.2.1 Inadequate caloric intake and assimilation (e.g., eating disorders, malabsorption)
b.2.2. Excessive caloric expenditure (e.g., excessive exercise)
b.2.3 Stress (e.g., environmental stressors, certain personality traits and psychological disorders)
b.2.4 Hypermetabolic states (e.g., severe infections, burns, traumatic brain injury, organ transplant and hyperthyroidism)
b.2.5 Age-related impairment of GnRH pulses
b.3 IATROGENIC
b.3.1 GnRH analogues
b.3.2. Drugs (e.g., sex steroids)
b.3.3 Pituitary irradiation, trauma or surgery
b.3.4 Opiates or alcohol abuse

amenorrhea and subsequent infertility (13). Considering the many possible causes, the clinical and hormonal profile of women affected by HH can be very heterogeneous. It has been assumed that, in patients with very low gonadotropins level, in addition to the stimulation with r-FSH a minimum threshold of serum LH is necessary to promote meiosis and final stages of antral follicular growth (14). In particular, the presence of an "LH therapeutic window" was hypothesized. According to this theory, the patients who benefit most from LH supplementation would be those with a mean baseline LH level equal to 1.0 IU/L. On the contrary, in women with LH levels higher than 1.7 IU/L, LH supplementation was deemed to be ineffective (15).

In affected women with an intact pituitary function, pulsatile GnRH therapy can be used to restore the periodic release of FSH and LH, resulting in ovulation. However, effective use of GnRH requires frequent administration (every 60–120 min) and the use of a portable pump injecting the drug either intravenous (iv) or subcutaneous (sc) for several weeks. The alternative therapeutic option is the administration of: i) human menopausal gonadotropin (hMG) (which contains both FSH and LH), ii) a combination of recombinant (r)FSH and recombinant (r)LH, iii) low doses of human chorionic gonadotropin (hCG) (16).

The first randomized clinical trial (RCT) designed to test the efficacy of rLH in HH women was conducted by the European Recombinant Human LH Study Group (16). Patients were randomly assigned to receive 0, 25, 75, or 225 IU rLH once daily in addition to 150 IU rFSH once daily for up to 20 days. Authors demonstrated that, in a dose-related manner, rLH promoted estradiol (E2) secretion, enhanced the effect of FSH on follicular growth, and permitted successful luteinization of follicles when exposed to hCG. In particular, patients who received 75 or 225 IU rLH were more sensitive to FSH than patients who received 25 IU or no rLH. Furthermore, authors observed that when FSH is administered alone to stimulate follicular development, E2 secretion is minimal, resulting in deficient endometrial growth. In addition, when exposed to hCG, these follicles frequently fail to luteinize. Importantly, the group that received 225 IU rLH had a smaller number of growing follicles when compared with the group who received 75 IU rLH. This could suggest an LH ceiling effect, whereby some secondary follicles underwent atresia due to their high sensitivity to LH (17). Loumaye et al. confirmed this effect showing that rLH when administered alone can trigger follicular growth arrest (18). The optimal rLH daily dose has been questioned also by a subsequent prospective, randomized, parallel-group, multicenter trial (15). In this small study, authors provided evidence suggestive of an LH threshold: follicular development was suboptimal when less than 75 IU/day rLH was administered (15).

The distribution and terminal half-lives for rLH are approximately a quarter those of rFSH when administered intravenously or given subcutaneously. Considering the differential pharmacokinetic and pharmacodynamic properties of both molecules, one may thus infer that the administration of rLH at narrower and repeated time intervals could be helpful to reduce serum gonadotrophin fluctuations between dose administrations, potentially improving drug accumulation and serum LH steady-state concentration (16). To untangle this issue, Awwad et al. conducted a non-randomized controlled pilot study aimed at investigating whether split daily doses of rLH was more efficacious than the single daily dose in supporting follicular development and ovulation in primary HH (13). Twenty-seven women with HH received a 150 IU fixed daily subcutaneous dose of rFSH, supplemented by 75 IU daily dose of rLH given either as a single dose (n = 9; single-dose group) or as four equally divided doses (n=18; split-dose group). Although lacking statistical significance, the proportion of women in the rLH split-dose group who fulfilled all three end points (i.e., at least one follicle ≥ 17 mm in diameter, pre-ovulatory serum E2 ≥ 400 pmol/l and a midluteal progesterone ≥ 25 nmol/l) was higher than the single-dose group (72.2% versus 55.6%). There were no serious untoward side effects. Authors concluded that administering rLH in split daily doses could provide superior results compared with the traditional single daily dose (13). The statistical power of the study is limited and additional evidence would be needed. On the other hand, split dose is not considered 'patient friendly' and, not surprisingly, no other researcher has further investigated this issue.

Some years later, Shoham et al. conducted a RCT in 25 medical centers in 4 countries. Patients with HH who desired pregnancy were randomized to receive either 75 IU rLH and 150 IU rFSH, or placebo and 150 IU rFSH. Results showed that 16 out of 24 patients treated with rLH and rFSH achieved follicular development compared with 2 out of 10 patients receiving placebo (p = 0.023) (19). Which exogenous source of gonadotropins was the most effective in HH women has also been a matter of debate. Carone et al. compared the efficacy of rFSH/rLH in a 2:1 ratio with highly purified hMG (hMG-HP) urinary extract in women affected by WHO type 1 anovulation. Included patients were randomly assigned to receive either 150 IU hMG-HP (150 IU FSH + 150 IU LH-like activity) (n=18 women) or 150IU rFSH + 75IU rLH daily (n=17 women) for a maximum of 16 days. Following a total of 70 cycles, 70% of rFSH/rhLH treated patients met the primary endpoint (i.e., at least one follicle ≥ 17 mm in diameter, pre-ovulatory serum E2 ≥ 400 pmol/l and a midluteal progesterone ≥ 25 nmol/l) versus 88% in the hMG-HP group (p=0.11). However, pregnancy rate in the rFSH/rLH group was 55.6% compared to 23.3% in the hMG-HP group (p=0.01) (20). Data published by Carone et al. were also re-analysed in a public health perspective: rFSH + rLH generated an incremental cost effectiveness ratio (ICER) equal to €2,007.30 compared to hMG-HP and the average cost per pregnancy was estimated to be €3,990.00 for recombinant strategy and €5,439.80 for urinary strategy (21).

The few data about the safety profile of both rFSH/rLH combination and hMG are reassuring. Further comparative

studies are warranted to investigate the tolerability, acceptability, and other adverse events, such as the risk of ovarian hyperstimulation syndrome of rFSH/rLH compared to the conventional hMG regimens to stimulate HH patients (17).

LH deficiency induced by GnRH analogue protocols

In the 1980s, the introduction of GnRH analogues revolutionised the efficacy of assisted reproductive techniques (ART). In fact, the so-called 'downregulation protocols', thanks to their ability in preventing the endogenous LH surge, reduced the rate of cycle cancellation, improved the ART outcomes and enabled some flexibility in scheduling oocyte retrieval (22). The administration of GnRH antagonist during OS determines a rapid and significant fall in LH levels. Usually, the residual hormone is enough to support steroidogenesis in theca cells, and rFSH is sufficient for OS (2). However, in a minority of patients, the 0.25 mg GnRH antagonist daily dose may determine an excessive decrease in LH concentration or a failure in rapidly restoring it (23). A history of ovulatory disorders and previous *in vitro* fertilization (IVF) antagonist cycle treatment seem to be associated with a higher risk of GnRH antagonist hyper-response (23). The impact of such a profound LH suppression on pregnancy outcomes is still debated (23).

GnRH agonists, after an initial increase in LH and FSH secretion (flare up), induce downregulation of the GnRH receptor. A long GnRH-agonist down regulation is thus responsible for a severe reduction of LH secretion (10). The impact of such a decrease in LH serum levels on reproductive outcomes is still a debated and unsolved issue with some studies demonstrating an association between a profound pituitary suppression and lower pregnancy and live birth rates and others denying it (24–27). Furthermore, the different LH threshold values used among studies to define low LH groups further complicate the interpretation and synthesis of available data (2).

Progestins recently emerged as alternatives to GnRH analogues. In fact, they were shown to strongly inhibit the pulsatile GnRH and LH secretion. Progestins are considered an effective option when a fresh embryo transfer (ET) cannot be performed (i.e., fertility preservation, anticipated hyper responders, preimplantation genetic testing (PGT), oocyte donors, etc.) (28, 29). Even after the administration of progestins, in subgroups of patients an excessive suppression of LH secretion may occur.

Against this background, several authors speculated a beneficial effect of LH supplementation in women treated with GnRH analogues. Results of studies investigating this issue are summarized in the next paragraphs. This hypothesis could also be considered valid in protocols involving the administration of progestins but, to date, no studies have yet tested it.

GnRH antagonist protocol

Several RCTs have been designed to investigate whether the addition of exogenous LH to a GnRH antagonist stimulation protocol could improve the ovarian response and, consequently, pregnancy rates (30–39). Mochtar et al. pooled their results in a Cochrane meta-analysis and found no clear evidence of a difference between rLH/rFSH and rFSH alone in terms of IVF success rates (9). Alviggi et al., in a more recent systematic review, confirmed the absence of a beneficial effect of combined treatment (10). Data syntheses have been criticized for the heterogeneous characteristics of the included populations. In particular, it has been speculated that older women being more prone to develop LH deficiency after GnRH antagonist could be the only ones to benefit from rLH supplementation. Studies published so far, appear statistically homogeneous but differ in some potentially determinant methodological aspects such as the use of oral contraceptive pill (OCP) the cycle prior to OS and the day of OS from which rLH was started. Bosch et al. administered rLH from the beginning of ovarian stimulation after one complete OCP cycle and demonstrated a significantly higher implantation rate in the study group (40). On the contrary, protocols adding rLH from stimulation day 6 did not demonstrate any improvement in IVF cycle outcomes in women 35 years and older. These findings reinforce the concept that the possible beneficial effect of LH requires that its administration starts concomitantly with FSH to achieve optimal steroidogenesis and a better oocyte competence. This role might be especially needed when an OCP is given in the cycle preceding OS, since it determines a marked reduction of LH serum concentration. Although tempting, this hypothesis needs a robust formal confirmation before it can be considered valid for clinical practice (40, 41).

GnRH agonist protocol

Six RCTs investigated the role of rLH supplementation in women who underwent pituitary suppression with long GnRH agonist protocols (42–47).

Their results are conflicting and prevent from definitive conclusions. Ferraretti et al., conducted the first RCT in the field and observed that the addition of a small amount of rLH to rFSH was associated with significantly higher chances of embryo implantation and pregnancy (42). The same research group, in a subsequent contribution, tested a new stimulation protocol consisting in a sequential administration of 150 IU rLH for 4 days followed by 400 IUI rFSH after downregulation with GnRH agonist. Interestingly, they observed that LH pretreatment was able to decrease the cancellation rate, to improve the *in vitro* performance, and to significantly increase the live birth rates (38). Matorras et al., on stimulation day 6, randomized women

aged 35-39 years to receive r FSH alone rFSH+rLH for the remaining ovarian stimulation period. In the 'intention to treat' (ITT) analysis, authors observed a significantly higher implantation and live birth rate in the group of women treated with rFSH+rLH. To note, these findings were not confirmed in the 'per protocol' (PP) analysis (40). On the other hand, both Tarlatzis et al. and Musters et al. failed to demonstrate a benefit in terms of IVF outcomes associated with the addition of r LH during the late follicular phase of a long GnRH agonist protocol (41, 43). A still debated aspect in GnRH agonist protocols is which LH source is most effective. Orvieto et al., critically presented the available evidence comparing the effect of the two commercially available LH preparations (hMG versus rFSH/rLH) on OS characteristics and in IVF cycle outcomes (48). Authors analysed the results of 10 studies adopting GnRH agonist protocols (three prospective studies of which two RCTs (49, 50) and one prospective observational study (51) and seven retrospective studies (52–58)). Data synthesis showed a higher number of oocytes retrieved but a lower rate of metaphase II (MII) oocytes and lower ongoing pregnancy and live birth rates in women treated with rLH when compared to women treated with hMG. However, the differences failed to reach statistical significance. The author thus established that no firm conclusions can be drawn in favor of a particular source of preparation containing 'LH activity' and that large RCTs are needed to confirm the true effect of the source of LH supplementation on IVF outcome (45). More recently, Kirshenbaum et al., in a cross-sectional study, compared OS outcome of two commercially available preparations with different source of LH bioactivity: rFSH/rLH in a fixed 2:1 ratio (Pergoveris®, Merck, Darmstadt, Germany) and HP-hMG, containing urinary FSH and LH activity provided by hCG in a fixed 1:1 ratio (Menopur®, Ferring pharmaceuticals). Patients treated with rFSH/rLH yielded significantly higher numbers of mature oocytes and fertilized oocytes, with non-significantly lower pregnancy rate per transfer (15% vs 29%, respectively), compared to those treated with HP-hMG (59).

Advanced maternal age women

Female ageing is characterized by the progressive increase of fully glycosylated FSH variants with a lower affinity for the FSH receptor when compared with the most common isoforms expressed in younger women (2). At the same time, the LH isoforms become progressively more sialylated and less sulfonated over time (2). The impairment of gonadotropins' action results in reduced steroidogenesis with negative repercussions on ovarian physiology. It was speculated that this form of age-related functional hypogonadism could be corrected or, at least, mitigated by the exogenous LH administration which is expected to increase the androgenic and estrogenic follicle fluid levels. Alviggi et al., summarized the results of RCTs testing this hypothesis and concluded that rLH exerts a beneficial effect in terms of implantation rate in women aged 36-39 years and has no impact in women ≥ 40 years (10). In a subsequent meta-analysis focused on women aged between 35 and 40 years, the same group of researchers demonstrated a positive association between rFSH/rLH cotreatment and clinical pregnancy rate (OR 1.45, 95%CI, 1.05-2.00, p=0.03) (60). However, the only two RCTs reporting the impact of rLH supplementation on the chances of live birth failed to demonstrate any benefit (OR 1.53, 95%CI, 0.50-4.65, p=0.45) (60). As recognized by the authors themselves, available evidence is insufficient and further data is needed. Future research initiatives should focus on narrower age ranges (i.e., 35-37 and 38-40 years) and more homogeneous populations (60). A limitation of data published so far regarding the impact of LH supplementation in advanced maternal age women is the inclusion of studies with relevant methodological differences. Among these, the main one concerns the type of OS protocol adopted. In fact, it is well known that the timing of onset of hypogonadotropic hypogonadism depends on the type of GnRH analogue used. Researchers interested in this issue should keep these methodological aspects in mind when designing future study protocols.

Genetic variants in gonadotropins' receptors: Pharmacological implications

LH acts through LH/HCG receptor (LHCGR). LHCGR is expressed on theca cells and, subsequently, develops on granulosa cells (16). Genetic variants of both LH and its receptor can alter the ovarian response to gonadotropins. Such conditions are usually diagnosed following unexpectedly poor responses to OS. Carriers of a common variant of the LH beta chain (rs1800447) are characterized by a less active form of LH that is not able to adequately support FSH activity during the stimulation of follicles' growth and, as a consequence, results in a reduced response to OS (2, 61). Interactions between FSH receptor (FSHR) and LHCGR polymorphisms are crucial in determining the response to OS protocols. Alviggi et al. observed that the presence of allele C on both FSHR-min29 (rs1394205) and LHCGR-291 (rs 12470652) was associated with an increased ratio between the cumulative rFSH consumption and the total number of oocytes as well as mature oocytes (relative risk (RR) 5.47; CI 95%, 3.13–7.81, p < 0.001) (62).

Lindgren et al. reported that women homozygous for LHCGR N312 required lower doses of exogenous FSH for adequate ovarian response. Considering the dimerization hypothesis, this could indicate that asparagine (N) is associated with a higher receptor sensitivity (63). They also studied the interaction between receptors' polymorphisms and showed that women homozygous for serine (S) in both considered polymorphisms (FSHR N680S polymorphism and LHCGR N312S polymorphism) had a 4-fold higher chance of

pregnancy compared with women homozygous for N in corresponding codons (63). A subsequent cross-sectional study indirectly confirmed Lindgren's finding (64). In fact, authors found that women heterozygous (N/S) or homozygous (S/S) for serine showed a higher requirement for rLH compared to those homozygous for asparagine (N/N) during OS. Moreover, in the same study, the pregnancy rate was significantly higher in serine carriers who received rFSH/rLH than in those receiving rFSH alone (64). These data combined with others (65) suggests a probable benefit of administering LH supplementation to women undergoing IVF on the basis of their single-nucleotide polymorphism profile (rs2293275) of LHCGR.

Discussion

The available evidence supports supplementation with exogenous LH for the treatment of WHO group I anovulatory women seeking pregnancy. The choice of the LH daily dose as well as the FSH : LH dose ratio are of utmost relevance in this group of patients. In fact, on the one hand, both an insufficient LH daily dose and a fixed 1:1 ratio were associated with a suboptimal follicular growth. On the other hand, it is equally important not to exceed the proper daily dose to avoid the so called 'ceiling effect'. Published data suggest that the ideal LH daily dose should be 75 IU (15, 17, 18). However, these recommendations cannot be applied to all patients and under all treatment circumstances. In fact, a daily LH dose of 25 IU is probably insufficient whereas one of 225 IU is associated with a higher risk of follicular atresia, with little being known about intermediate doses. As for the FSH : LH ratio, much of the literature suggests it should be equal to 2:1. However, also this aspect has not been still completely clarified.

These uncertainties should be the incentive for new investigations aimed at establishing criteria that could be useful for the personalization of treatment in this understudied WHO anovulation group.

The efficacy of supplementation with LH in advanced maternal age patients as well as in cases of depletion of FSH and LH levels induced by GnRH analogues has not been demonstrated. Again, further research efforts are needed. In fact, there is a strong suspicion that the discrepancy between what is suggested by the underlying theory (i.e., that iatrogenic HH could benefit from LH supplementation) and what emerged from previous data (9, 10) may be affected by methodological weaknesses as well as by the inhomogeneity of included populations. Age is probably a confounding factor. Indeed, one can speculate that advanced maternal age women are at increased risk of developing HH after GnRH analogues administration. Studies conducted so far have investigated the effect of LH supplementation in women who were considered prone to develop HH without, however, assessing serum LH levels before starting therapy. In GnRH antagonist protocols, attention should be given to those individuals whose LH level increases during the first half of the follicular phase. In fact, such endogenous hormonal trend during the first half of ovarian stimulation could be associated with a sharp decrease in LH immediately after the first GnRH antagonist injection, lack of LH level recovery 24 h later, and, consequently, need for compensation with exogenous LH (23). The proportion of the population selected for IVF experiencing this "abnormal" LH dynamic is probably not negligible. In fact, Kol et al. estimated that 33% of the patients have increased LH level during the first half of OS and are thus at higher risk of hyper-response to the first GnRH antagonist injection (23). The evaluation of serum LH levels should therefore be the cornerstone of future studies' design.

Data on gonadotropin receptor genetic variants have yet to be considered preliminary. However, this is a fascinating and promising area of research. If the receptor affinities associated with the different polymorphisms were confirmed on a large scale, a new chapter would open in the study of the personalization of OS therapy (2).

The detailed analysis of the other patient categories in which the efficacy of LH supplementation has been investigated (i.e., women with a hyporesponse to exogenous FSH monotherapy and women classified as poor responders to ovarian stimulation (10)) is beyond the scope of this narrative review. In this context, it is however important to underline that patients with these characteristics should not be included in studies aimed at investigating the benefit of LH supplementation on HH to avoid the interposition of confounding factors. The present review has some limitations that need to be acknowledged. First, even though the search of studies was conducted meticulously, the present review cannot be considered compliant with the official guidelines for systematic reviews. Second, we did not carry out a quantitative synthesis of available data. This also prevents us from providing information regarding statistical heterogeneity between studies. On the other hand, it must be recognized that the amount of data on the subject that could be pooled are still scarse and, therefore, the evidence provided by a possible meta-analysis on the subject would have a very limited reliability.

Conclusions

Knowledge regarding the efficacy of LH supplementation in HH patients has been accumulating in recent years. For the results to be considered reliable and useful in clinical practice, however, a methodological effort is required. First, it is necessary to focus on women with proven low LH serum levels or with failure in rapidly recovering LH concentrations after GnRH analogues administration. Second, one should control the population as much as possible for confounding factors either by designing randomized trials or by applying stringent inclusion criteria (i.e., narrow age ranges, predicted normal response to OS, homogeneous dose and source of exogenous gonadotropins, etc.). Third, as suggested by Bosch and colleagues (2), studies should include

information on endocrinological outcomes. In fact, assessing endocrine parameters such as serum testosterone, serum E2 and the E2/oocyte ratio could help in clarifying, in descriptive studies, the endocrinological profile during OS of women with depleted FSH and LH serum levels and, in intervention studies, the stimulatory effect exerted by LH during OS on steroidogenesis in both theca and granulosa cells.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Epigenetics of functional hypothalamic amenorrhea

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Functional hypothalamic amenorrhea (FHA) is a temporary infertility characterized by the suppression of the hypothalamic–pituitary–gonadal (HPG) axis, induced by the inhibition of the hypothalamic pulsatile secretion of the gonadotropin-releasing hormone (GnRH), in the presence of stressors, including eating disorders, excessive exercise, and psychological distress. Although the stressful factors that may lead to FHA are well-established, little is known about the inter-individual variability in response to stress and the consequent inhibition of the HPG axis. Not all women, indeed, manifest FHA in presence of stressful conditions. Recent studies highlighted a genetic contribution to FHA. Rare or polymorphic variants in genes that control the development and/or function of GnRH neurons may contribute, indeed, to the adaptability of the reproductive axis to stress factors. Also epigenetic changes have been associated with different pathways involved in the HPG axis and therefore, take part in FHA and confer a personal predisposition to anovulation consequent to a stressful event, or represent biological markers of response to stress. This review summarizes recent advances in the identification of the contribution of (epi)genetics to FHA and to long-term complications of functional amenorrhea, and reports insights into the involvement of additional genetic loci in FHA development on the bases of the clinical and molecular overlap with other gynecological and/or psychological conditions. Finally, we describe the promising application of induced pluripotent stem cells (iPSCs) as a new approach to investigate the molecular pathways involved in FHA.

KEYWORDS

functional hypothalamic amenorrhea (FHA), epigenetics, susceptibility genes, anorexia nervosa, delayed puberty

Introduction

Functional hypothalamic amenorrhea (FHA) may be considered a natural protective mechanism in women against stressful events, that temporarily suppress reproductive functions when physical conditions are not suitable to sustain a pregnancy. FHA is characterized by the suppression of the hypothalamic–pituitary–gonadal (HPG) axis, induced by the inhibition of the hypothalamic pulsatile secretion of the gonadotropin-releasing hormone (GnRH), in the presence of stressors, including eating disorders, excessive exercise, and psychological distress. FHA thus delineates as a temporary infertility characterized by the absence of menses for at least 3-6 consecutive months in absence of pregnancy, hyperandrogenism, hyperprolactinemia or other endocrine dysfunctions.

Although the stressful factors that may lead to FHA are well-established, little is known about the inter-individual variability in response to stress and the consequent inhibition of the HPG axis. Studies in women identified a high variability in the occurrence of FHA after heavy training. In particular, the frequency of FHA in athletes ranges from 6 to 43% (1); while in non-athletes, following the same training scheme of athletes, the frequency of FHA has been reported to be higher, with only about 14% of women with regular menstrual cycles during the training (2). Studies in nonhuman primates also confirmed the variable response of the HPG to stress (3–5). Like women, female macaques exposed to a combination of stresses showed different degrees of anovulation. Some animals, considered stress-sensitive, have an immediate suppression of ovulation and menstrual cycles, whereas others are highly stress-resilient and never experience FHA in presence of stress (3). Immunohistochemistry analysis of hypothalamic sections from resilient and stress-sensitive monkeys highlighted a higher GnRH expression in the neuron soma and lower GnRH levels in the neuron fibers of stress-sensitive animals, suggesting differences in the neuronal mechanisms involved in GnRH synthesis, transport and release in stress-sensitive compared with resilient animals (5).

Taken together, these studies suggest that the genetic variability in genes that control the development and/or function of GnRH neurons may contribute to the adaptability of the reproductive axis to a stressful condition. This hypothesis is confirmed by recent studies that highlighted the presence of a higher frequency of rare variants in genes associated with idiopathic hypogonadotropic hypogonadism (IHH) in women with FHA compared to women with regular menses (6, 7). The identification of other pathways, comprising those involving ghrelin and leptin that control the HPG axis as endocrine mediators of energy balance, could suggest the classification of FHA as a multifactorial and complex condition.

In addition to the presence of rare variants and polymorphisms that may alter the regulation of the axis, epigenetic changes could be associated with different pathways involved in the HPG axis (8, 9) and therefore, take part in FHA and confer a personal predisposition to anovulation consequent to a stressful event, or represent biological markers of response to stress.

In this review we summarize recent advances in the identification of the contribution of (epi)genetics to FHA and to long-term complications of functional amenorrhea, and reports insights into the involvement of additional genetic loci in FHA development on the bases of the clinical and molecular overlap with other gynecological and/or psychological conditions. Finally, we describe the promising application of induced pluripotent stem cells (iPSCs) as a new approach to investigate the molecular pathways involved in FHA.

Genetics and molecular overlapping between FHA and IHH

The clinical overlapping between FHA and IHH prompted the hypothesis that mutations in genes involved in IHH may confer susceptibility to the functional deficiency of GnRH secretion, the hallmark of FHA. IHH is, indeed, characterized by the failure to activate the pulsatile secretion of GnRH or the defective action of GnRH at pituitary level, thus resulting in absence of puberty and infertility. IHH is classified in IHH associated to anosmia, also known as Kallman syndrome (KS), a mendelian condition characterized by locus heterogeneity, that is found in about the 60% of IHH patients (10, 11); and normosmic IHH (nIHH) observed in the remaining patients.

The presence of total or partial loss of olfaction is caused by the common embryonic origin and developmental pathways of GnRH and olfactory neurons. Similar to olfactory fibers, GnRH neurons originate in the nasal placode from where they migrate to the hypothalamus (12). This common developmental pathway accounts for the defective migration of both GnRH and olfactory neurons in KS. The identification of the underlying molecular mechanism causative of KS, and the genetic characterization of affected families, allowed the identification of mutations in genes involved in the migration of GnRH and olfactory neurons, such as *KAL1* and *FGFR1* (13). More recently, mutations in the *PRORK2* and *PROK2* genes, that encode for a G-coupled receptor and its ligand with a fundamental role in the development of GnRH neuronal progenitors, have also been associated to IHH (14). Other genes that are involved in the development of IHH include: i) *CHD7*, encoding for a chromodomain protein associated to the CHARGE syndrome, suggesting a link between IHH, chromatin remodeling and transcription regulation; ii) *KISS1* and *KISS1R*, that encode for

kisspeptin 1 and its endogenous receptor, that are the most potent regulators of GnRH secretion in humans; iii) *LEP*, that encodes for leptin, a fat-released hormone that regulates food intake, energy expenditure and fertility at the hypothalamic level, and its receptor LEPR, thus linking body weight with the reproductive capability; iv) *TAC3* and *TAC3R*, that encode for neurokinin B and its receptor, suggested to have a role in GnRH secretion; v) *PCSK1*, encoding for the neuroendocrine convertase 1 (NEC1), which converts inactive peptides to bioactive molecules, including the adrenocorticotropin (ACTH); and vi) *GnRHR*, that is the main regulator of GnRH secretion.

Rare variants in IHH-related genes in FHA

After the identification of FHA in some families with KS/nIHH, Caronia and colleagues found six pathogenic variants in genes associated with IHH development in patients with FHA (Table 1 and Figure 1), thus suggesting, for the first time, an involvement of rare variants in these loci in the predisposition to FHA in presence of stressful situations (6). The study included 55 women with FHA and highlighted six heterozygous variants in *FGFR1*, *PROKR2*, *GNRHR* and *KAL1* in 7 patients with FHA and at least one predisposing factor to secondary amenorrhea. Four of these patients also reported a family history of FHA, but the genetic analysis was not extended to other affected relatives to confirm the association. The observed variants were considered pathogenic, since they all were located in conserved aminoacidic residues and induced a significant loss of function (6). None of these mutations were observed in a group of 422 control women, some of them also exposed to one risk factor for FHA (i.e., training for more than 5 hours a week). In addition, one mutation in the *PROKR2* gene (L173R) and in *GNRHR* were also detected in a cohort of 160 IHH patients.

The same authors further deepened the role of genetics in FHA, extending the mutational analysis to 53 genes involved in IHH and the number of FHA patients included in the study (7). This mutational screening allowed the identification of 78 heterozygous variants in 58 out of 108 women with FHA (Table 1 and Figure 1). Among these, three variants were validations of the previously smaller cohort, 32 novel variants were identified in patients resulted negative at the previously sequencing analysis, and two were detected in a patient with a previously identified mutation in the *ANOS1* gene. Most of these variants were observed in FHA women only, while few of them were also found in control women, thus excluding a pathogenetic role of these variants per se in the development of FHA. However, the frequency of rare variants in GnRH-related genes was higher in FHA patients compared to controls, and 35% of variants observed in amenorrhoeic women has been previously associated with IHH development, compared to 19% found in the control group (7). In addition, FHA women harbor a higher number of variants (three or more) compared to the control group. In this latter many participants did not show any variant. In this study, no associations with the number of FHA risk factors nor with the family history for the condition were observed (7).

Overall, this evidence further supports the involvement of genetic variants in the variable response of the reproductive axis to stress. Moreover, since only mutations in *FGFR1* and *CHD7* are known to be associated to IHH with a dominant inheritance, the authors hypothesized that the GnRH deficiency may be considered as a semi-dominant disease, with heterozygous carriers that show a milder phenotype, characterized by FHA only in presence of triggering external factors, while patients with biallelic or di/oligogenic mutations show a severe form of GnRH deficiency resulting in IHH. This is supported by the identification of some rare variants also in eumenorrheic women. These studies thus suggest, for the first time, that FHA may be part of a disease spectrum with a genetic base.

Genetic and molecular overlapping between FHA and other gynecological disorders

Several other disorders, including delayed puberty and anorexia nervosa, share with FHA a partially overlapping clinical presentation and the causative molecular mechanisms, suggesting a common genetic base or predisposition. This common picture stems from both the central role of the hypothalamus, which controls most of the basic functions that are interconnected, and the multifunctional role of the hormones released by the hypothalamus. In particular, genetic variants in genes involved in pathways controlling appetite or stress-response might also contribute to FHA. Anorexia nervosa (AN), for example, is a multifactorial eating disorder characterized by a chronic energy deficiency that leads to the suppression of the HPG axis because of the reduced secretion of GnRH, as observed in FHA. AN and FHA enter in differential diagnosis since AN may be considered a cause of functional amenorrhea. Differently from FHA, AN shows a strong heritability since 1) family studies have demonstrated a significant prevalence of AN in first-degree relatives of probands compared to controls, with a 11.3 times more probability of relatives to develop AN compared to controls (15, 16); 2) studies on twins highlighted that monozygotic twins have a higher concordance rate of AN development compared to dizygotic twin, with an estimated heritability of 88%; 3) population studies reported an estimated heritability of approximately 58% for AN, with the remaining variance associate with environmental factors (17); 4) mutational screenings and genome-wide association studies have

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TABLE 1 Rare variants in IHH-related genes identified in FHA patients according to Caronia et al., 2011 and Delaney et al., 2020.

Gene	Protein activity	Role in HPG axis	Variants
FGFR1	Tyrosine kinase receptor	GnRH neuron migration/development	Arg756His
			Gly260Glu
PROKR2	GPCR	GnRH neuron migration/development	Arg85His
			Leu173Arg
			Thr340Ser
			Met111Arg
GNRHR	GPCR	GnRH action	Arg262Gln
			Ser168Agr
			Gln106Arg
KAL1	Cell adhesion	GnRH neuron migration/development	Val371Ile
RAB3GAP2	Neurotransmitter exocytosis	Neurodevelopmental syndrome with IHH*	Asp1206Tyr
			Pro527Leu
			Arg420Cys
			Leu1331Ile
RAB3GAP1	Neurotransmitter exocytosis	GnRH neuron migration/development	Arg336Cys
			Arg954His
HESX1	Transcriptional repressor	Pituitary gland development	Val129Ile
SOX2	Transcription factor	Pituitary gland development	Gly22Ser
KLB	Membrane receptor	GnRH neuron migration/development	Ala169Thr
			Gly908Val
			Lys815Glu
			Val1042Ile
TACR3	TAC3 receptor	GnRH secretion	His248Arg
OTUD4	De-ubiquitylating enzyme	Neurodevelopmental syndrome with IHH^	Pro933Arg
SRA1	Regulator of nuclear receptors	Steroid activity	Leu110Aspfs*25
SPRY4	Inhibitor of MPAK receptor	GnRH neuron migration/development	Ser241Tyr
			Gly92Val
			Cys209Tyr
PROP1	Transcription factor	Pituitary gland development	Ala142Val
SEMA3E	Growth factor	GnRH neuron migration/development	Pro171Ser
			Asn153Ser
			Asp580Asn
GNRH1	Gonadotropin-releasing hormone	GnRH secretion	Ile48Arg
FGFR1	Growth-factor receptor	GnRH neuron migration/development	Gly291Glu
CHD7	Chromatin-remodeling factor	GnRH neuron migration/development	Ser244Arg
			Arg459Cys
			Asp728His
			Pro1705Gln
			Arg1942Trp
			Met2527Leu
			Met396Ile
			Ser466Leu
			Leu2984Phe
			Met340Val
LHX3	Transcription factor	Pituitary gland development	Gly317Ser
			Arg315Pro
WDR11	Transcription factor	GnRH neuron migration/development	Val6Met
POLR3B	Subunit of RNA polymerase III	Neurodevelopmental syndrome with IHH°	Lys721*

(Continued)

TABLE 1 Continued

Gene	Protein activity	Role in HPG axis	Variants
			Arg978Cys
KL	Ligand of KLB	GnRH neuron migration/development	Arg751Gly
			Val845Gly
DMXL2	Synaptic protein	GnRH neuron migration/development	Met563Val
			Thr476Se
			Ile2573Val
			Ile1317Val
DCC	Transmembrane receptor	GnRH neuron migration/development	Gly470Asp
			Asp819Asn
			Val883Ile
			Asn635Ser
PNPLA6	Phospholipase	Neurodevelopmental syndrome with IHH^	Gly1329Arg
AXL	Tyrosine kinase receptor	GnRH neuron migration/development	His292Profs*47
			Gly517Ser
			Val289Met
FLRT3	Cell adhesion/signaling	GnRH neuron migration/development	Gln401Leu
ANOS1	Extracellular glycoprotein	GnRH neuron migration/development	His672Arg
			Val587Leu
			Val371Ile
			Ser511Tyr
NR0B1	Nuclear receptor	Adrenal gland development	Ser412Gly
LEPR	Leptin receptor	Neuroendocrine regulation	Val754Met
CCDC141	Cytoskeletal-associated protein	GnRH neuron migration/development	Glu876Lys
PCSK1	Protease	Pituitary gland development	Thr640Ala

Underlined variants have been observed also in eumenorrheic women.
*, Warburg Micro syndrome/Martsolf syndrome; ^, Gordon Holmes syndrome; °, 4H syndrome.

highlighted several genetic loci that may account for the predisposition to AN (18). Common variants in neurotrophin signaling genes, including *BDNF*, *NTRK2* and *NTRK3*, seem to contribute to the susceptibility to eating disorders (19, 20) (Table 2). Other signaling pathways, involving a cross-regulation of satiety and estrogen production, including the serotoninergic and leptin pathways, have been linked to an increased risk to develop AN. Common variants in the *OPRD1*, *HTRD1*, *EBF1* and *SLC6A4* genes show a higher frequency in AN patients compared to controls and segregate in family members with AN, supporting their involvement in the etiology of AN (21–24) (Table 2). Common or rare variants in genes involved in these pathways have never been investigated in FHA patients. However, given the close link between FHA and AN, in addition to the evidence that food restriction is one of the main risk factors for FHA development, it can be hypothesized that variants in genes involved in satiety, appetite and weight regulation may play a role in the differential response to physical and/or psychological stressors and the consequent inhibition of the HPG axis in FHA.

Delayed puberty may occur in patients with FHA and IHH and can be considered an early clinical sign of these conditions. About two-third of FHA patients report a family history of delayed puberty with an apparent autosomal dominant pattern of inheritance, sometimes with incomplete penetrance (25, 26). Genome-wide association studies highlighted an association with genes involved in IHH, including *LEPR*, *GNRH1* and *TACR2* (27, 28) (Table 2). Besides, genetic screenings in IHH families identified mutations in *HS6ST1*, *FGFR1* and *KLB* in IHH patients and their relatives manifesting delayed puberty only (29–31) (Table 2). Pathogenic mutations in the *IGSF10* gene were also found in six unrelated families with delayed puberty in a large Finnish cohort of patients (32) (Table 2). *IGSF10* mutations affect the migration of GnRH neurons from the vomeronasal organ in the nose to the forebrain during embryonic development, possibly affecting the predisposition to FHA as observed for other genes in the same pathway.

All this evidence supports a common genetic basis of FHA with AN, delayed puberty and IHH, strengthening, again, the hypothesis that monoallelic mutations in a subset of genes that modulate the HPG axis may lead to AN, delayed puberty and/or FHA, while biallelic mutations or a specific combination of heterozygous variants in these genes could lead to a more sever phenotype, including IHH or KS (33).

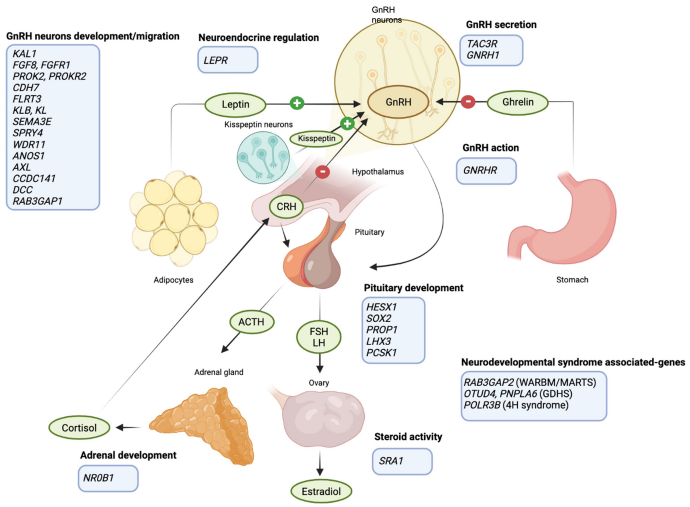

FIGURE 1
Schematic representation of the HPG axis regulation and the FHA predisposing genes. GnRH neurons in the hypothalamus release GnRH upon different stimuli: kisspeptin, produced by a specific group of hypothalamic neurons, is a major player in the neuroendocrine control of GnRH and gonadotrophins secretion; ghrelin and leptin allow the regulation of GnRH secretion according to energy balance (link between HPG axis and food intake); cortisol inhibits GnRH secretion (link between HPG axis and anxiety). FHA-predisposing genes are listed in the light blue boxes (created with BioRender.com).

TABLE 2 Predisposing genes to gynecological and psychological conditions showing overlapping features with FHA, and to long-term consequences of FHA.

Disorder	Condition	Overlapping with FHA	Affected pathway	Genes involved
Gynecological disorders	Anorexia nervosa	AN is a chronic energy deficiency that leads to the suppression of the HPG axis because of the reduced secretion of GnRH	Neurotrophin signaling pathway	*BDNF* *NTRK2* *NTRK3*
			Serotoninergic and leptin pathways	*OPRD1* *HTRD1* *EBF1* *SLC6A4*
	Delayed puberty	Delayed puberty may occur in patients with FHA and can be considered an early clinical sign of this condition	IHH development	*LEPR* *GNRH1* *TACR2* *HS6ST1* *FGFR1* *KLB*
			GnRH neuron migration	*IGSF10*
Psychological disorders	Anxiety	The neuroendocrine response to stress and stress-related neuronal plasticity involves the HPG axis	Energy balance and anxiogenic effect of CRH	*NPY*
	Mood disorders	Altered neuroplasticity related to stress	Neuroplasticity, neurogenesis, neuronal survival, and differentiation	*BDNF*
Long-term consequences	Osteopenia and osteoporosis	Prolonged hypoestrogenism in FHA leads to osteopenia and osteoporosis	Estrogen receptor	*ESR1-XbaI*
			Vitamin D receptor	*VDRBsmI site* *VDRFokI site*

Genetic and molecular overlapping between FHA and psychological disorders

FHA may be considered a multifactorial disease with genetic factors and pathomechanisms overlapping not only with other gynecological conditions, but also with psychiatric traits, since an anxious behavior is considered a main sign of FHA. The hyperactivation of the hypothalamic–pituitary–adrenal (HPA) axis in presence of stressing factors is, indeed, a typical feature of FHA and this is believed to be one of the most important pathogenetic factors in FHA patients (34, 35). Increased corticotropin-releasing hormone (CRH) secretion results in an augmented secretion of adrenocorticotrophin from the pituitary, and cortisol from the adrenal glands that, in turn, leads to reduced GnRH secretion.

Several studies have highlighted a strong genetic component in anxiety predisposition or resilience to stress. Genome-wide association studies identified several common genetic variants in genes involved in the neuroendocrine response to stress and in neuronal plasticity that affect stress perception and increase the risk of developing stress-related disorders. Some of these polymorphisms are in genes playing a role also in controlling the HPG axis, thus suggesting a possible link among stress-associated genetic variants, FHA, and anxiety. In particular, specific polymorphisms (i.e. rs16147 and rs3214187) in the *Neuropeptide Y*, which acts as a regulator of energy balance and counteracts the anxiogenic effect of CRH (36) (Table 2), have been associated to a resilience or a stress-sensitive phenotype in presence of a stressful condition (37, 38). The NPY also controls GnRH concentration, by inducing its release in presence of adequate levels of estradiol. In hypoestrogenic women, NPY inhibits GnRH release and amenorrheic patients show lower levels of basal serum NPY (39, 40). NPY may, thus, act as a link between the HPG and HPA axis, and common or rare variants in the *NPY* gene may affect the HPG axis response to stress (Figure 2).

Neuroplasticity has a primary role in the predisposition to stress-related disorders. According to the neurotrophic hypothesis, mood-disorders may be associated with impaired structural plasticity and cellular resilience, with a key role of the brain derived neurotrophic factor (BDNF), that is highly expressed in the hippocampus and involved in neuroplasticity, neurogenesis, neuronal survival, and differentiation (41). Several

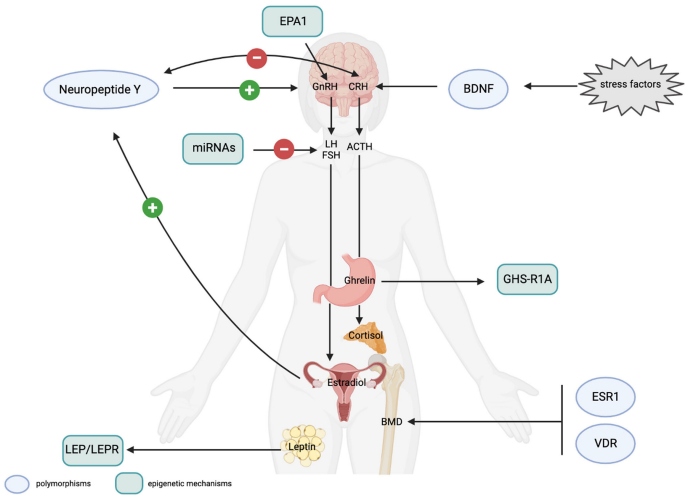

FIGURE 2
(Epi)genetic mechanisms possibly involved in FHA development and FHA-related long-term consequences. **Genetic mechanisms**. Polymorphisms in the *Neuropeptide Y* (*NPY*) and *BDNF* genes affect stress response. The NPY positively controls GnRH secretion in presence of adequate levels of estrogen and has an anxiolytic effect by counteracting CRH activity. CRH, itself, downregulates the expression of the NPY. NPY polymorphisms have been associated to resilience or stress-sensitive phenotypes. BDNF polymorphisms are suggested to affect neuroplasticity and stress responses. Polymorphisms in the estrogen receptor (ESR1) and in the vitamin D receptor (VDR) genes influence bone mineral density (BMD) and may be associated to osteopenia and osteoporosis, consequent to prolonged hypoestrogenism. **Epigenetic mechanisms**. The EPA1 transcription factor controls GnRH expression and a 5'-UTR polymorphism has been associated with a higher risk of amenorrhea in animal models. Altered methylation levels of the *LEP* and *LEPR* genes have been associated with the effect of leptin, produced by adipocytes on the HPG axis and on the personal response to psychotherapeutic treatment in AN patients. Methylation of the ghrelin receptor gene (*GHS-R1A*) are thought to be involved in ghrelin resistance affecting GnRH secretion. Specific miRNAs have been reported to control the post-transcriptional expression of LH and FSH, and to be a promising peripheral biomarkers to control the effect of hormonal therapy in FHA women. Light blue circles indicate polymorphic variants in genes possibly associated to response to stress or long-term consequences in FHA women; light green rectangles indicate the epigenetic mechanisms (including transcription factors, miRNA and methylation) that can play a role in the regulation of the HPG axis and in FHA development (created with BioRender.com).

studies have highlighted an increase of BDNF serum levels associated with stress and mood disorders (42). Genome-wide association studies pointed for the involvement of the Val66Met (rs6265) in anxiety and response to stress, with Val/Val homozygotes that show increased anxiety (43), suggesting a role of BDNF in gene-environment interactions (Table 2). Despite the function of BDNF in FHA is not fully understood, a significant lower concentration of plasma BDNF was found in FHA patients in comparison to healthy controls (44), possibly suggesting the contribution of BDNF in the altered stress response associated to FHA (Figure 2), paving the way for the analysis of common variants in the BDNF gene that may account for the anxiogenic phenotype of FHA women.

Genetic predisposition to long-term complications of FHA

FHA is a complex clinical condition, that should be addressed with a multidisciplinary approach.

The close interconnections between the hormonal and neuroendocrine status to regulate homeostasis, lead, consequently, to clinical manifestations other than amenorrhea in FHA women. The final endocrinological consequence of GnRH impairment is, indeed, hypoestrogenism. Persistent low levels of estrogen have negative effects on different aspects of female health. In particular, normal estrogen levels are fundamental in young women for bone metabolism, the correct function of the cardiovascular system, and for mental health. Considering these pleiotropic effects of hypoestrogenism, genetic studies on amenorrheic patients should also include the genotyping of candidate loci associated with long-term complications of FHA.

Among these, prolonged hypoestrogenism in young women with FHA is associated with osteopenia and osteoporosis. Studies in women with AN highlighted the association of specific polymorphisms with bone mineral density (BMD). In particular, the A allele at a polymorphic site of the estrogen receptor alpha ESR1-XbaI (rs934079) has been associated with a reduced BMD (45) (Table 2); while the AA genotype at the VDRBsmI site (rs1544410) and the CT genotype at the VDRFokI site (rs2228570) of the vitamin D receptor (VDR), both show a positive correlation with BMD in patients with AN (46) (Table 2). According to this evidence, the identification of FHA women at higher risk for reduced BMD should be mandatory for a timely treatment and to avoid long-term consequences of FHA. Polymorphic variants in ESR receptors may also be investigated in association to other FHA-related health consequences, including cardiovascular disorders primarily derived from endothelial dysfunction secondary to hypoestrogenism (47) (Figure 2).

Serum sex steroid levels in women also control mood. Low levels of estrogen in FHA women are strongly coupled to the modulation of the activity of different neuropeptides and neurotransmitters, in particular serotonin and dopamine, affecting mood in amenorrheic patients (48).

Mood disorders and anxiety are both causes and effects of FHA, thus establishing a negative loop that exacerbates the effects of mood disorders and sustains FHA. As previously described, many variants have been identified in genes associated with a personal response to stress and estrogen sensitivity that influence mood disorder predisposition. The identification of genetic variants in these or other genes in the same pathways may allow the identification of FHA patients who are more predisposed to mood disorders. This approach could improve a tailored therapeutic approach based on targeted therapies to avoid or disrupt the loop between FHA and mood disorders.

The epigenetic contribution to FHA

Epigenetic mechanisms govern gene expression without changing gene sequence and comprise chromatin changes, DNA methylation, and the expression of non-coding RNAs. The epigenetic signatures can be modified in response to environmental factors, stress, or disease, and mediate the response of the organism to external factors. Importantly, they can be reverted by epigenetic drugs, as demonstrated in cancer, by changes in behavioral habits or non-pharmacological treatments, such as psychotherapy.

Recent evidence highlighted the importance of the epigenetic regulation of GnRH expression by a network of miRNAs, epigenetic modifications, and transcription factors, suggesting an important role of these mechanisms in regulating the HPG axis and, therefore, their possible involvement in FHA. The tight interplay between genes and environment in FHA development and the identification of a few genetic variants associated to FHA predisposition, suggest that epigenetic factors may represent additional candidates underlying FHA pathogenesis. Recent studies on mice have reported a pivotal role of epigenetics in controlling GnRH neuron ontogenesis, through the coordinated actions of *Dnmt3b*, *Tet1* and *Ezh2* on the *Fgf8* transcription (49). Moreover, *in vitro* studies further highlighted that the GnRH gene responds to external stimuli by modulating chromatin modifications also in mature GnRH neurons (50). This evidence suggests that epigenetics is a major molecular regulator of GnRH neuron development and function, and that the deregulation of these mechanisms may affect the activity of the HPG axis and the reproductive capacity.

Several studies analyzed the epigenetic profiling in amenorrheic women, mainly with AN, thus highlighting

several differentially methylated genes. In particular, *LEP* and *LEPR* methylation levels were lower in AN women compared to controls (51) (Figure 2). Besides, lower DNA methylation of the *LEP* gene was associated with a significant hypermethylation during psychotherapeutic treatment and full recovery in AN patients (51), thus suggesting a predictive value of *LEP* methylation in identifying patients with a higher probability of recovery after treatment (51). Also hypomethylation of the *GHS-R1A* gene, that encodes for the ghrelin receptor, has been reported in AN patients, supporting the effect of environment on satiety and ghrelin resistance (52), and a possible effect of this epigenetic alterations in FHA patients (Figure 2).

GnRH secretion is controlled also by the activity of specific transcription factors. In particular, EAP1 (Enchanced At Puberty 1) is a transcription factor with a dual activity on the GnRH gene: it activates GnRH transcription and, at the same time, inhibits the expression of the preproenkephalin, that represses GnRH secretion. A polymorphic site in the 5-UTR of the gene has been associated with a higher risk for amenorrhea in non-human primate models (53), thus linking the epigenetic control of GnRH with common genetic variants that confer a higher risk for FHA (Figure 2).

Recently, growing findings have unveiled the central position of miRNAs as key regulators of GnRH secretion and consequent pituitary activation. Some miRNAs (miR-132, miR-212, miR-361-3p) have been reported to be induced by GnRH (54, 55) and play a role in the gonadotropin pathways, by directly targeting the 3'-UTR of LH and FSH transcripts or downregulating the expression of specific transcription factors (56) (Figure 2). The expression profiling of miRNAs in peripheral blood is emerging as a promising tool to monitor the effect of hormonal therapy in FHA. Studies on animal models have, indeed, demonstrated that kisspeptin-based hormonal therapy stimulates gonadotropin secretion and the altered expression of specific miRNAs in plasma (57). *In silico* prediction of targeted transcripts highlighted that kisspeptin-induced miRNAs may affect cell transport, structural and functional cell polarity, neural networks and intracellular trafficking, in addition to DNA methylation and sphingolipid metabolism. These studies, thus, open new research venues to identify the involvement of miRNAs in FHA and their possible use as peripheral biomarkers to monitor the effect of therapies. Nonetheless, microRNAs are not in clinical use yet, mainly due to technological limitations, including the lack of assay standardization and reproducibility (58).

iPSCs to study the molecular mechanisms underlying amenorrhea

Most studies focused on the dissection of the neuroendocrine mechanisms leading to GnRH inhibition and FHA in presence of stress have been performed on animal models (5, 59, 60). However, this approach cannot fully recapitulate the complexity of the disease, given the physiological differences in estrous and menstrual cycles, the failure of reproduce in animal stressful conditions comparable to humans, and the higher complexity of response to stress in human that also encompasses self-awareness. In addition, since FHA is a multifactorial disease, animal models do not allow the comprehensive investigation of (epi)genetic and environmental factors associated with FHA. For these reasons, induced pluripotent stem cells (iPSCs) from FHA patients, that can be differentiated into GnRH neurons, are a promising tool to deepen the molecular bases of FHA, and *in vitro* investigate the effect of predicted predisposing variants and new therapeutic strategies.

In the last years, the generation of iPSC lines combined with the CRISPR–Cas9 technology has led to the generation and characterization of GnRH neurons, that are promising cell models to dissect signaling pathways and gene regulatory networks involved in human GnRH neuron development and function (61, 62). iPSCs from patients with AN have been already used to study changes in gene expression profiles that may occur in AN, highlighting that most of differentially expressed genes belong to the tachykinin receptor pathway and estrogen response (63), thus suggesting their possible application also to study the pathomechanisms underlying FHA. iPSCs may be also used to test therapies targeting not only GnRH release, but also the serotoninergic and dopaminergic neurotransmission, in order to improve the behavioral features of FHA, taking into account the personal response mediated by the epigenetic profile of each patient.

Conclusions

Recent evidence highlighted that the establishment of FHA is variable among women, because it is a complex disease whose phenotypic appearance is influenced by several factors, including stress, behavioral habits and (epi)genetics, that together impact on the regulation of the HPG axis.

For this reason, (epi)genetic variants possibly associated to the personal predisposition to FHA should be investigated

among genes that control the release and function of HPG-related hormones, and among genes involved in the interconnection between the environment and stress response.

The identification of rare genetic and epigenetic variants associated with FHA may have important clinical implications, as they may represent druggable targets for personalize medicine. In particular, germ-line variants may improve the clinical stratification of patients according to the patient- specific molecular profile, whereas epigenetic specific signatures, based on their dynamic nature, may represent valuable peripheral biomarkers for the diagnosis of the disease and for monitoring the effect of pharmacological and psychological therapies.

Author contributions

FL critically revised the literature and wrote the manuscript; GE, MG, and GV provided support for the clinical content of the manuscript and critically revised the review; MM supervised and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Bone health in functional hypothalamic amenorrhea: What the endocrinologist needs to know

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In the original definition by Klinefelter, Albright and Griswold, the expression "hypothalamic hypoestrogenism" was used to describe functional hypothalamic amenorrhoea (FHA). Given the well-known effects of estrogens on bone, the physiopathology of skeletal fragility in this condition may appear self-explanatory. Actually, a growing body of evidence has clarified that estrogens are only part of the story. FHA occurs in eating disorders, overtraining, and during psychological or physical stress. Despite some specific characteristics which differentiate these conditions, relative energy deficiency is a common trigger that initiates the metabolic and endocrine derangements contributing to bone loss. Conversely, data on the impact of amenorrhoea on bone density or microarchitecture are controversial, and reduced bone mass is observed even in patients with preserved menstrual cycle. Consistently, oral estrogen-progestin combinations have not proven beneficial on bone density of amenorrheic women. Low bone density is a highly prevalent finding in these patients and entails an increased risk of stress or fragility fractures, and failure to achieve peak bone mass and target height in young girls. Pharmacological treatments have been studied, including androgens, insulin-like growth factor-1, bisphosphonates, denosumab, teriparatide, leptin, but none of them is currently approved for use in FHA. A timely screening for bone complications and a multidisciplinary, customized approach aiming to restore energy balance, ensure adequate protein, calcium and vitamin D intake, and reverse the detrimental metabolic-endocrine changes typical of this condition, should be the preferred approach until further studies are available.

KEYWORDS

functional hypothalamic amenorrhea (FHA), female athlete triad, bone, osteoporosis, oral contraceptives (OCs), estrogen, anorexia nervosa

Introduction

Functional hypothalamic amenorrhea (FHA) is a condition of chronic hypoestrogenism without identifiable organic causes (1). It is encountered in undernutrition and eating disorders (e.g. anorexia nervosa, AN), overtraining, emotional stress and chronic diseases (2), and entails long-term consequences, including bone loss (1).

The definition of osteopenia and osteoporosis in AN is inconsistent among studies: while some have considered bone mineral density (BMD) T-scores (osteopenia: -1.0<T-score<-2.5; osteoporosis: T-score<-2.5), others have reported z-scores and defined osteopenia at -1.0<z-score<-2.0 and osteoporosis at z-score<-2.0. Overall, osteopenia is reported in 25-90% and osteoporosis in 19-44% of adult women with AN (3–6). More than a half of AN adolescent girls present z-score<-1 at one or more sites, most commonly the spine (7). In amenorrheic AN women, BMD declines by 2.4% at the hip and by 2.6% at the spine annually (8).

Impaired microarchitecture (9–13) and bone strength (9) have been documented in AN, resulting in a cumulative incidence of fragility fractures up to 57% (14). Fracture risk is increased at all ages and at several sites, particularly the hip, pelvis, spine and distal forearm (15, 16).

Women with exercise-related FHA have low BMD, even though to a lesser extent than AN patients (17). The "Female Athlete Triad" is a condition characterized by low energy availability, FHA, and osteoporosis (18). Z score<-2.0 and -1.0<z-score<-2.0 have been reported in 0-15.4% and 0-39.8% of female athletes respectively (19). This variability may result from the varied effects which different sports exert on bone (20). Amenorrheic athletes also have impaired microarchitecture (21, 22) and bone strength (22), and a higher risk of stress fractures (28-47%) compared to eumenorrheic athletes (17-25.6%) and nonathletes (23, 24).

When FHA manifests at young age, it irreversibly impairs bone mass accrual, since 90% of peak bone mass (PBM) is achieved by the age of 18 (25). Adult women with AN onset before age 18 show lower spine BMD than those developing it later, regardless of amenorrhea duration (26). Additionally, final height can be impaired (27) and bone maturation delayed (28). Despite weight and menstrual recovery, individuals who experience bone loss as adolescents have chronic deficits and an increased risk of fracture in adulthood (29–31).

In this review, we summarize determinants of bone loss, pitfalls in assessment and treatment, and indications for management of FHA-related skeletal fragility.

Determinants of bone loss

As a condition of estrogen deficiency, skeletal involvement may appear straightforward in FHA, since estrogens exert a predominantly antiresorptive action on bone (32) and, along with growth hormone (GH), insulin-like growth factor 1 (IGF-1) and energy balance, play a key role in pubertal growth and bone mass accrual (33).

Hypoestrogenism

Delayed menarche and longer amenorrhea duration are associated with low BMD, altered microarchitecture, reduced strength, and fractures in AN- (5, 26, 34), stress- (17) and exercise-FHA (22, 30, 35, 36). Age of onset is critical since estrogen deficiency during adolescence determines low PBM, which adds to hypoestrogenism-related bone loss during adulthood (26). However, the estradiol threshold considered to have skeletal effects in the general female population has classically been set at 25-30 pg/ml and further lowered to 5 pg/ml in subsequent studies (37), while serum concentrations are generally higher in FHA (17, 21). Additionally, reduced BMD is found in eumenorrheic AN women too, particularly at the hip (38). These observations suggest that hypoestrogenemia is not the only determinant of bone loss, and effects may vary at different skeletal sites. Indeed, Miller et al. documented low BMD at the spine, hip and radius in AN women, and only at the spine in normal-weight women with other forms of FHA (39). Spine BMD is significantly lower in amenorrheic athletes than in eumenorrheic ones (24, 35), while femoral BMD is comparable (35). Therefore, hypoestrogenism appears to impact mainly on trabecular (e.g. spine) bone, while other factors like body mass index, lean mass and mechanical load act on cortical (e.g. hip) bone (8, 34, 35, 38).

Lifestyle factors

Energy imbalance is the initial trigger for the (mal-)adaptive changes and comorbidities observed in FHA (40, 41).

Energy deficit yields changes in hypothalamus-pituitary axes, adipokines and gastrointestinal hormones which, in turn, affect bone (42, 43). Describing this neuro-endocrine adaptation is beyond the purpose of this review. However, in order to understand the rationale behind treatment approaches, the lifestyle and the hormonal contributors to bone disease are summarized in Figure 1 (42, 43).

Calcium and vitamin D intake, physical activity and lean body mass influence PBM (25). Untreated AN patients present lower circulating levels of 25OH-vitamin D and 1,25OH-vitamin D than controls (44). Vitamin D insufficiency (serum 25OH-vitamin D<30 ng/mL) is observed in more than 50% AN women and is associated with higher parathyroid hormone concentrations and lower hip BMD (45). Among adolescent female gymnasts, 83.3% present vitamin D insufficiency, 33.3% vitamin D deficiency (<20 ng/mL), and 72.2% a poor dietary calcium intake (46), and these factors are associated with impaired bone turnover (47).

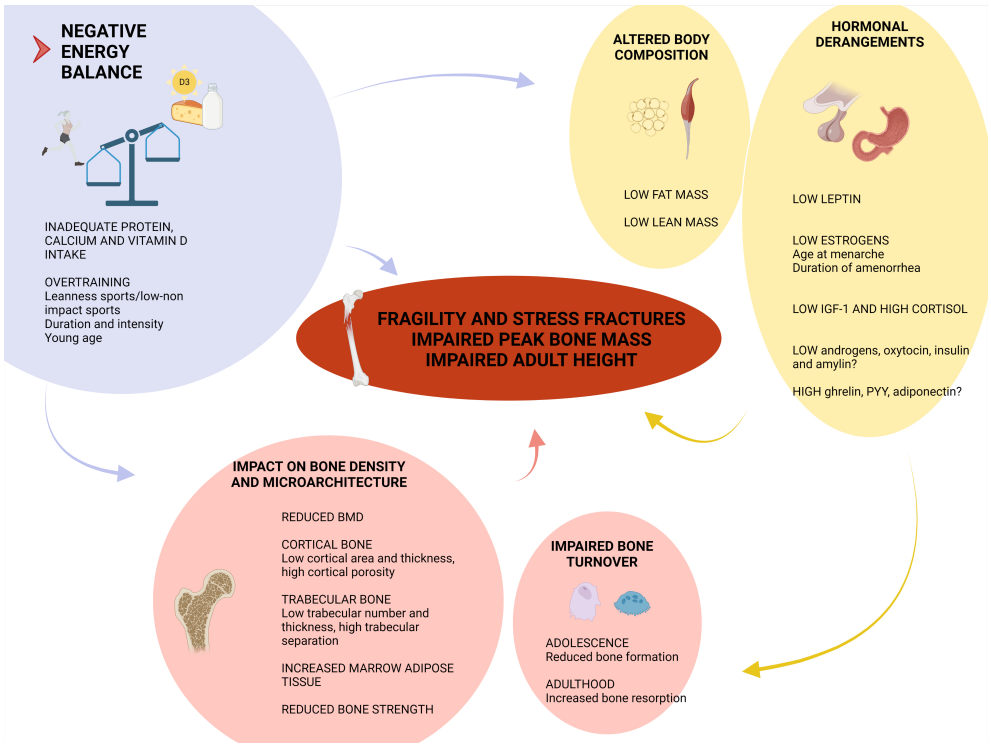

FIGURE 1
Determinants of skeletal fragility in functional hypothalamic amenorrhea (FHA). Negative energy balance is the prime determinant in FHA physiopathology. Not only the caloric intake, but also the insufficient amount of dietary calcium, vitamin D and proteins impact on bone. In case of overtraining, some features of sports, like mechanical load and exercise intensity, can affect bone health. The reduction in lean mass impairs peak bone mass achievement and cortical bone microarchitecture. The reduction in fat mass is associated with low leptin levels and hypogonadism. Estrogen deficiency contributes to the increased bone resorption (mainly observed in adulthood) and altered trabecular bone mineral density (BMD) and microarchitecture. The low levels of insulin-like growth factor-1 (IGF-1) result from growth hormone resistance and the nutritional deprivation, and participate in lowering bone turnover as observed in adolescent patients, and in disrupting peak bone mass achievement. The hypothalamus-pituitary-adrenal axis is overactive in FHA, resulting in enhanced cortisol secretion which, in turn, inhibits intestinal calcium absorption, increases urinary calcium excretion, inhibits osteoblast proliferation and increases marrow fat content. Further studies are needed to clarify whether testosterone, dehydroepiandrosterone, ghrelin, peptide YY (PYY), adiponectin, insulin, amylin and oxytocin play a role in FHA skeletal involvement (42, 43). (Created with BioRender.com).

The impact of exercise on bone is complex, since the entity of mechanical loading (48) and exercise intensity (49) influence bone metabolism and fractures and interact with energy and gonadal status. Activities with high or odd mechanical strain, like ball, power or antigravitational sports, induce bone mass gain and improve bone geometry and strength (20, 21, 50), particularly at weight-bearing sites. Conversely, in sports generating low or repetitive loading, like endurance running, ballet and swimming, the detrimental effects of energy deficit prevail (20, 51).

The effects of exercise in AN are controversial and vary according to exercise intensity, mechanical loading, and phase of illness. High bone-loading activities performed for 1-6 hours/week during recovery from AN, may enhance bone accrual. Conversely, low-mechanical loading activities performed for <1 or >6 hours/week increase risk of bone loss (52, 53).

Chronic diseases

Some conditions associated with FHA (54) -like HIV infection, organ transplant- can cause bone loss *per se*, and/or because of medications used (antiretrovirals, glucocorticoids) (55). Also some drugs, like some antidepressants, can directly contribute to both FHA and osteoporosis (55).

Table 1 summarizes determinants of bone loss in different FHA forms.

Skeletal evaluation

Bone density

Dual-energy X-ray absorptiometry (DXA) is used for evaluation of areal BMD. However, heterogeneous definitions

TABLE 1 Determinants of bone loss in different forms of functional hypothalamic amenorrhea.

Undernutrition / Eating disorders	Overtraining	Systemic diseases / psychological stress
Delayed menarche, amenorrhea Young age with impaired peak bone mass Inadequate calorie, protein and calcium intake Vitamin D insufficiency/deficiency Hyponatremia Low mechanical-loading physical activity (e.g. running) Low body weight with low fat mass and low lean mass Drugs: • Diuretics abuse • Selective serotonin re-uptake inhibitors Endocrine modifications: ↓Leptin ↓GnRH pulsatility, LH, FSH and estrogens ↑CRH, ACTH and cortisol ↑GH with GH resistance, ↓IGF-1 ↓Androgens ↓Oxytocin, insulin, amylin ? ↑PYY, ghrelin, adiponectin ?	Delayed menarche, amenorrhea Young age with impaired peak bone mass Relative energy deficiency Inadequate protein or calcium intake Vitamin D insufficiency/deficiency Possible coexistence of eating disorders Sports with low or repetitive loading (e.g. endurance running, ballet, swimming) Leanness sports Training intensity Low body weight with low fat mass Endocrine modifications: ↓Leptin ↓GnRH pulsatility, LH, FSH and estrogens ↑CRH, ACTH and cortisol ↓IGF-1 ↑Ghrelin ↑PYY ↑ or = adiponectin ↓Insulin ↓Oxytocin	Delayed menarche, amenorrhea Young age with impaired peak bone mass Underlying conditions and/or drugs: • Gastrointestinal malabsorption (inflammatory bowel disease, coeliac disease) • Chronic inflammatory diseases with hypermetabolic states (e.g. rheumatoid arthritis) • Organ failure (e.g. cystic fibrosis, chronic renal disease, liver disease) • Organ transplant and medications (e.g. glucocorticoids, immunosuppressants) • HIV infection and medications • Diabetes (types 1 and 2) • Depression and/or selective serotonin re-uptake inhibitors Endocrine modifications: ↓GnRH pulsatility, LH, FSH and estrogens ↑CRH, ACTH and cortisol Immune stress (IL-1β, IL-6, TNF-α)

GnRH, Gonadotropin-releasing hormone. LH, luteinizing hormone. FSH, follicle-stimulating hormone. CRH, corticotropin-releasing hormone. ACTH, adrenocorticotropic hormone. Up arrow, increase. Down arrow, reduction. =, no variation. GH, growth hormone. IGF-1, insulin-like growth factor 1. PYY, peptide YY. IL-1β, interleukin 1β. IL-6, interleukin 6. TNF-α, tumor necrosis factor α.

of low BMD in FHA have been used in research studies and by scientific societies.

According to the International Society for Clinical Densitometry (56), z-score rather than T-score should be considered in pre-menopausal women, for whom there are no densitometric criteria of osteopenia and osteoporosis. Instead, BMD is defined as "below the expected range for (chronological) age" if z-score is <-2 (57, 58). In adult women, a diagnosis of osteoporosis is established if secondary causes of low BMD or risk factors for fracture are present too (57, 58), while in adolescents a clinically significant fracture history is required (59, 60).

The definition of low BMD in the 2007 position statement of the American College of Sports Medicine, is substantially different: low BMD in premenopausal athletes is defined at -2<z-score<-1 (18), considering that sportswomen have 5–15% higher BMD than nonathletes. More recently, the Female Athlete Triad Coalition and the Endocrine Society differentiated weight-bearing and non-weight-bearing sports (1, 61). In the former case -1.0<z-score<-2.0 deserves attention; for other sports, low BMD is diagnosed at z-score<-2.

Physicians should bear in mind that z-score>−2.0 does not exclude skeletal fragility. In fact, BMD explains 60-80% of bone strength and does not encompass other skeletal features (62).

Bone quality

Trabecular bone score is a textural index that provides an indirect measurement of lumbar spine trabecular microarchitecture (63, 64). Trabecular bone score is impaired in a significant percentage of AN adolescents and may represent a useful tool for skeletal evaluation (64, 65).

High-resolution peripheral quantitative computed tomography allows characterization of volumetric BMD, bone geometry and microarchitecture. Studies with this technique documented decreased cortical area and thickness, higher cortical porosity, lower trabecular number and thickness, and increased trabecular separation, in AN patients (9, 11, 66) and in amenorrheic athletes (21–23). Patients with multiple fractures have the most significant microarchitecture deterioration (23).

Morphometric vertebral fractures

The prevalence of asymptomatic vertebral fractures in FHA is unknown. One study including 80 young AN women found a low rate of prevalent and incident morphometric fractures, which were not predicted by BMD, duration or severity of malnutrition (67). While the screening of asymptomatic vertebral fractures is recommended in primary and most secondary forms of osteoporosis (55), there is no such indication in FHA.

Biochemical markers

Bone formation and resorption markers are used as indicators of treatment efficacy and compliance (55). Although they are not indicated for routine patients' evaluation (55), they can help characterize the turnover status of an individual.

Energy and estrogen status affect turnover in a time-dependent manner: while adolescents with AN show mainly reduced formation (68), in adult women enhanced bone resorption prevails (69), resulting in uncoupling of bone metabolism.

Assessment of estradiol levels has poor diagnostic significance since menstrual periods reflect estrogen status. However, current guidelines suggest to collect this value (1), which may serve for the differential diagnosis with polycystic ovary syndrome (70) and the therapeutic decision-making in patients planning pregnancy (1). Conversely, usefulness in the diagnostic and therapeutic work-up of skeletal complications is not defined and is probably limited.

Lifestyle intervention

Evidence on lifestyle and pharmacological approaches is summarized in Table 2.

Correction of energy deficit, weight recovery and resumption of menses are primary goals, and the finding of low BMD may motivate patients towards behavioural changes (106). Positive energy balance can be achieved by reducing exercise energy expenditure and/or increasing caloric intake, according to published recommendations (107). However, a threshold level of weight or body mass index gain is not established (76). An experienced multidisciplinary team is advocated for the management of these patients, including nutritionist, psychologist or psychiatrist, athletic trainer, internist, sports physician (106).

Weight gain reverses uncoupling of bone remodelling by increasing bone formation and reducing bone resorption in the short- and middle-term (108, 109). Weight improvement and resumption of menses are associated with BMD stabilization or increase (8, 72–75), even if some studies reported conflicting results (77, 110, 111). A longitudinal study demonstrated normalisation of spine BMD, bone volume and volumetric BMD in adolescents 2.7 years after recovery from AN (112). However, some observations suggested that only a partial effect can be achieved with weight recovery alone without restoration of gonadal function (71, 75, 113), or at least that a differential effect is exerted by these two factors: in a study by Miller et al., BMD increased at the hip following weight gain, and at the spine following menstrual recovery (8).

An adequate intake of calcium and vitamin D is generally recommended to ensure bone health (55). No study has specifically addressed this issue in FHA (76, 78). However, hypovitaminosis D may counteract the efficacy of refeeding in AN (79). Therefore, recommendations on adequate calcium and vitamin D intake appear appropriate (76).

However, bone disease may be not completely reversible, as low BMD and increased fracture risk can persist lifelong after sustained recovery from FHA (14, 75, 82).

Pharmacological treatment

Estrogens

Trials with oral contraceptives (OCs) or hormonal replacement therapy have led to controversial results. Reduction in bone resorption but also formation markers has been reported with OCs in FHA (80, 81, 83). In AN women, OCs did not increase BMD significantly over 1 year compared with no treatment or placebo (84, 85), and osteopenia persisted or progressed after 3 years (84). Conversely, in women with other forms of FHA, two placebo-controlled trials reported improvement in spine, but not hip BMD after 1 year (83, 86). A 4-year sequential therapy with 17β-estradiol and dydrogesterone significantly increased BMD from baseline (87). According to other findings, OCs may even be detrimental for bone mass recovery: in an observational study, AN women receiving OCs showed no BMD improvement despite weight gain, whereas hip BMD increased in women who gained weight but did not receive OCs (8).

On the other side, hormonal replacement therapy (i.e. transdermal estradiol with cyclic progesterone) over 12-18 months yielded a significant increase in spine and hip BMD in adolescents and young women with FHA compared with placebo (88) or OCs (89).

The controversial efficacy of OCs, as opposed to the transdermal estrogen administration, is ascribed to the further lowering of IGF-1 and (free) androgens concentrations caused by the former (39) (Figure 1). In addition, the ethinyl-estradiol content of OCs has been progressively reduced to the minimum effective dose, because of concerns on thromboembolic events. Subsequently, conflicting results have been reported about the effects on bone of low-dose and very-low-dose OCs in young women (90–92).

Androgens

Two studies reported on the use of low-dose transdermal testosterone in AN. The first failed to find significant changes in bone formation markers versus no treatment (93) and the second did not document BMD improvement from baseline following 12-month therapy (94).

TABLE 2 Summary of evidence of lifestyle change and pharmacologic treatments.

Lifestyle change and pharmacologic treatment	Reference	Clinical evidence
Weight gain	Giollo et al. (71)	Increase in spine BMD by 1.1% over 20 weeks; no change in hip
	Mika et al. (72)	No change over 2 years
	Compston et al. (73); Gordon et al. (74)	No change over 1 year
	Viapiana et al. (75)	Increase in spine and hip BMD by 4.8 and 7.1% respectively over 15 months
Weight gain + menses restoration	Miller et al. (8)	Mean annual increase in spine and hip BMD by 3.1 and 1.8% respectively
	Misra et al. (76)	Stabilization of BMD measures over 9 months
	Dominguez et al. (77)	Increase in spine and hip BMD by 4.6 and 3.1% respectively over 2.2 months
Calcium and vitD supplementation	-	-
Oral contraceptives	Grinspoon et al. (78); Vescovi et al. (79)	Reduction in bone resorption and formation markers
	Golden et al. (80); Strokosch et al. (81)	No increase in BMD over 1 year vs placebo
	Warren et al. (82); Hergenroeder et al. (83)	Improvement in lumbar spine but not hip BMD over 1 year vs placebo
	Sowińska et al. (84)	Increase in BMD from baseline over 4 years
Transdermal estradiol	Misra et al. (85); Ackerman et al. (86)	Increase in spine and hip BMD over 18 months vs placebo
Androgens	Miller et al. (87)	No changes in osteocalcin and BALP levels after transdermal testosterone vs placebo
	Miller et al. (88)	No increase in BMD from baseline over 12 months transdermal testosterone
	Bloch et al. (89)	No increase in BMD after DHEA vs placebo
	Di Vasta et al. (90)	Stabilization of femoral neck BMD after DHEA + oral contraceptives vs placebo
IGF-1	Misra et al. (91); Grinspoon et al. (92)	Increase in bone formation markers vs placebo
	Grinspoon et al. (93)	Improvement in BMD after IGF-1 + oral contraceptives vs IGF-1 alone
	Fazeli et al. (94)	No changes in bone turnover markers after rh-GH
Bisphosphonates	Golden et al. (95)	Increase in femoral neck but not lumbar spine BMD after alendronate
	Miller et al. (96); Miller et al. (88)	Increase in spine BMD after risedronate
	Haines et al. (97)	Increase in spine BMD after risedronate + IGF-1 vs risedronate alone
Denosumab	Jamieson et al. (98)	Increase in spine, hip and femoral neck BMD
Teriparatide	Milos et al. (99); Shibli-Rahhal et al. (100)	Increase in femoral neck BMD
	Fazeli et al. (101)	Increase in lumbar spine BMD
Recombinant leptin	Welt et al. (102); Chou et al. (103); Foo et al. (104)	Increase in bone formation markers
	Sienkiewicz et al. (105)	Increase in lumbar spine BMD

BMD, bone mineral density. vitD, vitamin D. BALP, bone alkaline phosphatase. DHEA, dehydroepiandrosterone. IGF-1, recombinant human insulin-like growth factor 1. rh-GH, recombinant human Growth Hormone.

In AN women, two placebo-controlled trials found no increase in BMD at any site with dehydroepiandrosterone alone (95), and a stabilization of femoral neck BMD with dehydroepiandrosterone given in combination with an OC for 18 months (96).

IGF-1

Short-term therapy with recombinant human IGF-1 increases bone formation markers in AN patients compared to placebo (97, 98). Improvement in BMD is observed when IGF-1 is given in combination with OCs (101). Supraphysiological recombinant human GH administration does not affect turnover markers in AN women (99).

Bisphosphonates

Femoral neck, but not spine BMD increased from baseline after 1 year of treatment with alendronate (100), while risedronate improved spine BMD compared to placebo when administered for 1 year in AN women (94, 102). A sequential

therapy with IGF-1 followed by risedronate increased spine BMD more than risedronate alone (103).

Denosumab

Experience with denosumab is anecdotal. Increase in spine and hip BMD has been reported in a woman with AN treated with denosumab for 3 years (104).

Teriparatide

Three studies reported a positive effect of teriparatide on spine BMD after 6 months (105) and on femoral neck BMD after 2 years (114, 115) compared to placebo in women with AN and severe osteoporosis.

Recombinant leptin

Leptin administration for 3-9 months in women with FHA improves gonadal, thyroid and growth hormone axes function, bone formation markers, RANK-ligand/osteoprotegerin ratio (116–118), and spine BMD after 2 years compared with no treatment (119). However, weight loss was observed in leptin-treated patients (119), making this treatment unsuitable for low-weight women with FHA.

Discussion

FHA is responsible for 20-35% cases of secondary amenorrhea (120). Some forms of FHA can take a long time to recover (121), and all bring about long-term health consequences (120). In addition, FHA involves a wide range of hormonal changes which differentiate it from other conditions of estrogen deficiency and make its management challenging.

The first step in the work-up of FHA-related bone loss, consists in selecting patients for clinical evaluation. Current guidelines recommend to screen patients with FHA lasting ≥6 months, or earlier if other risk factors occur, including low body weight, eating disorders, delayed menarche or prior fractures (1, 61). DXA scan of lumbar spine, hip and, in adolescents, whole body, is recommended for BMD evaluation, while parameters like trabecular bone score and bone microarchitecture should be reserved to research purposes. Only z-score has to be considered in pre-menopausal women, and low BMD is defined as z-score<-2 (1, 61). DXA scan has to be repeated every 12-24 months in patients at risk for bone loss and to monitor treatment (1, 18, 61). As no recommendation exists about screening for asymptomatic vertebral fractures, we suggest that the decision is left to clinical judgement, according to the presence of other risk factors or comorbidities contributing to skeletal demineralization (55).

Studies on pharmacological treatments have led to no definitive conclusions. Level of evidence is low, as based on few randomized controlled trials of short duration, or observational studies including small cohorts. Moreover, effects have been evaluated in terms of BMD or turnover markers, while data on fracture prevention are lacking.

Based on FHA-related hormonal changes, treatments with estrogens, IGF-1, leptin, androgens have been attempted. However, one single hormonal therapy has no or little effect on the other mechanisms which influence bone metabolism.

Administration of OCs has led to conflicting results. In addition, physicians should consider that resumption of menses through estrogen prescription could reduce patient's motivation to gain weight (122). Nevertheless, it is estimated that up to 78% of physicians prescribe OCs inappropriately to prevent bone loss (122).

Bone active medications are promising alternatives but more data are needed before including them in treatment recommendations. Bone active drugs should be prescribed cautiously in young women of reproductive age, considering that they cannot be administered for long periods, some of them raise concerns about fracture risk after discontinuation (123, 124), and the long half-life of bisphosphonates.

Both the Female Athlete Triad Coalition and the Endocrine Society suggest prescription of transdermal estradiol with cyclic progestins (but not OCs) in high-risk patients (i.e. z-score<-2.0, prior fractures) who did not respond to 1 year of non-pharmacological therapy (i.e. further BMD loss or new fracture) (1, 61). While the Endocrine Society recommends against the use of other medications (1), the Coalition suggests to consider bone active agents in women with contraindications to or lack of benefit from estrogen replacement (61).

At present, the cornerstone of management of FHA-related bone loss is lifestyle intervention. Weight gain has the most robust impact on BMD, and recovery of gonadal function has an additive effect (125). Patients should be aware that normalization of energy balance is the main factor that anticipates resumption of menses and BMD gain; however, achieving these goals takes months or years. Regular monitoring and long-lasting support should be provided by an experienced multidisciplinary team (18).

Nutritional intervention should address caloric intake and micro- and macronutrients availability. Since no data are available on the optimal daily intake of calcium and vitamin D in FHA, recommendations for other forms of osteoporosis are adopted (55, 78), i.e. 1000–1300 mg of calcium and 400–800 IU of vitamin D daily, eventually through oral supplements (18, 61). Serum 25OH-vitamin D levels should be monitored, aiming at concentrations of 30-50 ng/ml. Protein intake should be customized considering that intensely training athletes have higher requirements (18).

Energy availability can be increased by reducing exercising intensity too. This should be planned with a sports physician and athletic trainer for female athletes. In AN, moderate physical activity may be acceptable during the recovery phase (52, 53).

Lifestyle intervention must continue also in patients who receive pharmacological treatments.

In conclusion, FHA is a condition of estrogen deficiency which entails several metabolic and hormonal alterations. Bone is severely affected, with long-term consequences including short stature, reduced PBM and skeletal fragility. While re-establishing adequate energy balance and nutrients intake is instrumental to weight gain, recovery of menses and resolution of hormonal derangements, bone impairment is not completely reversible and the increased fracture risk can persist life-long. Preventive educational programs should be undertaken in schools or during athletic training. Larger and longer-lasting trials, new therapeutic approaches and combined strategies are warranted to improve bone health.

Author contributions

RI, VL, EF searched and selected the scientific literature and prepared the manuscript. GM and MA critically revised the manuscript. All authors approved the submitted version and agree to be accountable for the content of the work.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Genetic and phenotypic differences between sexes in congenital hypogonadotropic hypogonadism (CHH): Large cohort analysis from a single tertiary centre

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Background: Congenital hypogonadotropic hypogonadism (CHH) is a condition with a strong genetic background, caused by a deficient production, secretion, or action of gonadotropin-releasing hormone (GnRH). Published data on CHH cohorts indicate a male predominance, although this is not supported by our current understandings.

Aims: In order to unravel the possible causes or contributors to such epidemiological sex difference, the aim of our study is to investigate differences in genetic background and clinical presentation between males and females in a large cohort of CHH patients.

Materials and methods: We enrolled 338 CHH patients with absent or arrested pubertal development, referred to our Center from 01/2016. Data collection included clinical assessment at diagnosis and genetic analysis performed by next generation sequencing (NGS), employing a custom panel of 28 candidate genes.

Results: Among 338 patients 94 were female (F) and 244 male (M), with a ratio of 1:2.6. We found that 36.09% (122/338) of patients harbored potentially pathogenic rare genetic variants (RVs) with no significant differences between sexes; on the other hand, a significantly higher frequency of oligogenicity was observed in females (F 9,57% 9/94 vs M 3,69% 9/244, P = 0.034). The prevalence of non-reproductive phenotypic features was significantly higher (P = 0.01) in males (53/228, 23.2%) than in females (10/93, 10.8%): in particular, kidney abnormalities affected only male patients and midline defects had a significantly higher prevalence in males (P = 0.010). Finally, BMI SDS was -0.04 $\pm$ 1.09 in females and 0.69 $\pm$ 1.51 in males, with a statistically significant difference between groups (P = <0.001).

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Conclusion: Our data confirm the male predominance in CHH and identify some differences with regard to the clinical presentation between males and females that could indicate a variable expression of genetic rare variants and a dimorphic modulation of phenotype according to metabolic/behavioral factors, which will need to be substantiated and investigated by further studies.

KEYWORDS

congenital hypogonadotropic hypogonadism (CHH), genetics, female, GnRH (gonadotropin releasing hormone), phenotype [mesh], sex

Introduction

Human pubertal development and reproduction is under the control of the hypothalamus-pituitary-gonadal axis, whose master regulator is represented by the GnRH-secreting neurons. Regular functioning of these neurons is the result of a sophisticated, complex and interconnected network which integrates different central and peripheral signals (Figure 1) (1). A failure in the correct development and/or activation of the human GnRH-secreting neurons lead to a congenital GnRH-deficiency named Congenital Hypogonadotropic Hypogonadism (CHH). CHH is a rare and complex disease characterized by a GnRH deficient production, secretion, or action. Despite its pathogenesis has not been completely understood yet, several evidence from familial pedigrees and animal models suggest a strong genetic background. Currently, more than sixty genes have been associated with CHH; nonetheless, as much as 50% of cases remain without an identified genetic cause (2). CHH can be clinically associated or not with a defective sense of smell, identifying, respectively, Kallmann syndrome (KS) and normosmic CHH (nCHH). Once accounted as two entirely separate diseases, KS and nCHH are now largely considered as different manifestations of the same genetic disease, since they often coexist in the same kindreds and they partly share the same genetic milieu (3). With the exception of ANOS1 mutations in hemizygosity, which are almost invariably associated with a defective sense of smell, mutations of genes involved in GnRH neuron migration, neuron fate specification or differentiation have been associated with variable degrees of olfactory defects, with either KS or nCHH phenotypes. Instead, rare variants of CHH genes associated with impairment of GnRH neuron activation or action are responsible for nCHH only (4).

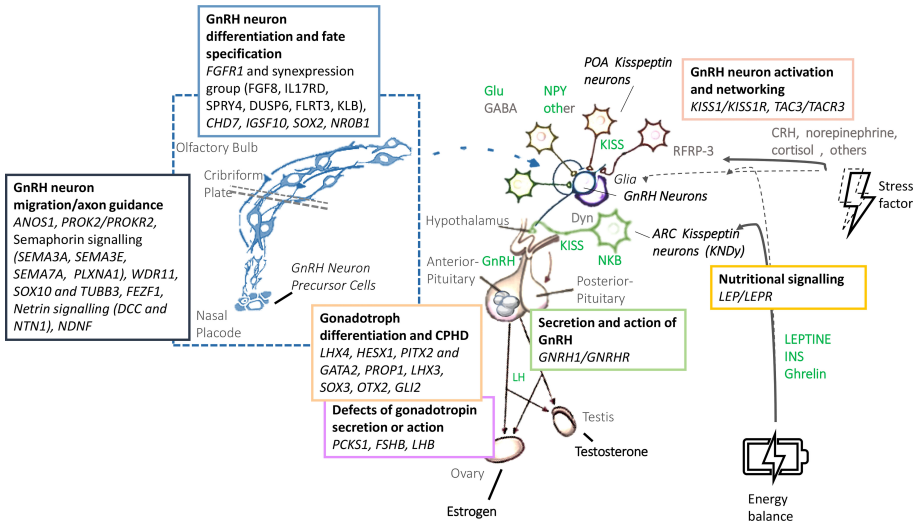

FIGURE 1
Biology and genetic background of the GnRH neuronal system. GnRH, gonadotropin-releasing hormone; LH, Luteinizing Hormone; FSH: Follicle-Stimulating Hormone; Glu, glutamate; GABA, gamma-amino-butyric acid; NPY, neuropeptide Y; POA, preoptic area; KISS, Kisspeptin; NKB, neurokinin B; Dyn, dynorphin; CRH, cortitropin releasing hormone; ARC, arcuate nucleus; INS, insulin.

Over the last fifty years, different studies have screened an ever-increasing number of candidate genes for CHH thanks to the use of Next Generation Sequencing (NGS) techniques, demonstrating an escalating impact of oligogenicity, which contributes to explaining the apparent variable penetrance and expressivity of some variants. However, since NGS allows the simultaneous screening of a wide number of genes, it also identifies a large number of rare variants of uncertain clinical significance (VUS). As pointed out in a recent survey among Expert Centers of the European Reference Network for rare endocrine conditions (ENDO-ERN) (5), the challenge is therefore to identify truly pathogenic variants more reliably, and distinguish true oligogenic inheritance from incidental rare findings that are not related to CHH.

The true prevalence of CHH is limited by the scarcity of published literature. In the original study the prevalence was estimated in 1:4,415 males (6), whilst more recently a Finnish retrospective study described an incidence of KS of 1:48,000, 1:30,000 in males and 1:125,000 females (7).

Indeed, CHH is traditionally considered a male predominant condition, with a male-to-female ratio of 4-5:1 (8–10), although when familial cases were analyzed separately, the ratio dropped to 2.3:1 (10). More recently there has been a reassessment of such imbalance, with the latest studies conducted among large patient cohorts reporting a male to female ratio of 3.6:1 (11) and 2.7:1 (12) and the sex ratio of affected individuals is closer to be equal in CHH kindreds (13, 14).

Nonetheless, the epidemiological sex imbalance, although apparently less consistent than previously considered, is not supported by the current understanding of CHH genetic basis. Indeed, only 3.5–10% (7, 15, 16) of cases harbor *ANOS1* mutations with a recognized X-linked inheritance.

The aim of this study is therefore to investigate possible differences in genetic background and clinical presentation in a large cohort of CHH patients, to unravel the possible causes or contributors to the observed epidemiological difference between males and females. An analysis of the female population will also be conducted to shed light on genotypic and phenotypic features.

Materials and methods

Study Population

We evaluated 338 CHH patients (94 females and 244 males, age at diagnosis 16.86 ± 3.11) who were referred to our Academic Medical Centre to perform genetic investigations and were therefore consecutively recruited from January 2016 to December 2021. Relevant patients' data were retrospectively assembled as part of routine clinical practice based on the delivery of good clinical care, accomplishing the Declaration of Helsinki. The study was approved by the Ethic Committee of the coordinating institution (GR-2008-1137632), and all patients (or their parents/guardians) gave a written informed consent. All subjects were affected with pre-pubertal CHH, defined as the lack of complete spontaneous pubertal development. CHH was diagnosed as (1): manifestations of hypogonadism and delay/arrest of puberty associated with low testosterone/estradiol and inappropriately low/normal gonadotropins (2); absence of any known acquired cause of hypogonadotropic hypogonadism (i.e., expansive hypothalamic/pituitary lesions, hemochromatosis, etc.), or multiple pituitary hormone defects (MPHD). In order to remove the functional hypothalamic defects, the exclusion criteria were: (I) severe weight loss or eating disorder (17); (II) intensive exercise (>12 hours/week); (III) chronic illness and psychiatric disorders. Both patients with either a normal sense of smell or olfactory defects (hypo- or anosmia), as demonstrated using Brief Smell Identification Test (B-SIT) and/or MRI, were included. Anonymous patients' data at the time of diagnosis, before starting any hormonal treatment, were retrospectively collected and a clinical database was created. Each patient's genetics and disease phenotype were also reported.

Phenotypic characterization

The age at diagnosis and the presence of any reproductive and/or non-reproductive phenotypical feature associated with CHH (the so called "red flags": anosmia, cryptorchidism, microphallus, deafness, kidney abnormalities, midline defects and bimanual synkinesis) were recorded for each patient. The presence of anosmia allowed for a diagnosis of KS, and was considered separately from the other features. The presence of family history (defined as a history of pubertal delay, confirmed secondary hypogonadism in the absence of other obvious causes, or reproductive and nonreproductive defects associated with CHH in relatives up to the second degree) was also recorded. In addition, the segregation pattern of rare variants was recorded, whenever available. Moreover, anthropometric parameters (height, weight, and body mass index (BMI) were recorded in 50/94 (53.19%) females and 116/244 (47.54%) males. Standard deviation scores (SDS) according to the WHO age curves were obtained using Growth Calculator 3.0, in order to compare different reference standards between sexes. In addition, Tanner stages (18) at diagnosis for mammary development (Breast Tanner Stage) and pubic hair (Pubic hair Tanner Stage) in females, and for genitalia and pubic hair in males, were evaluated. Finally, uterine length (as in the longitudinal diameter of the uterus assessed on pelvic ultrasonography) was measured in females. All data were collected prior to any hormonal treatment.

For the female population we recorded the hormonal investigations carried out at diagnosis: basal LH, FSH and estradiol ($17\beta E_2$) determination and dynamic testing with GnRH analogue. In particular, the LHRH stimulation test was performed using a standard protocol that involves taking basal venous blood samples for FSH and LH (0'), the subsequent

intravenous administration of LHRH Ferring 0.1mg/1ml 100 μg, and the collection of blood samples at 30', 60', 90' and 120 minutes for FSH and LH.

Due to the retrospective design of this study and the need to consider all values at diagnosis, different methods of hormonal measurement have been used. Nonetheless, in most cases, serum LH, FSH and 17βEstradiol concentrations were measured by electrochemiluminescence immunoassay "ECLIA". These LH and FSH assays have a lower limit of detection of 0.1 IU/L, while estradiol assays usually have a lower limit of detection of 5 pg/mL. For the purposes of statistical analysis, LH and FSH values below the lower reference limit were estimated as 0.1 U/L, while 17βE2 values below the lower reference limit were estimated as 5 pg/ml.

Genetic analyses by targeted next generation sequencing (NGS)

Each patient underwent a genetic investigation, using a targeted NGS technique, to look for rare allelic variants. We extracted the genomic DNA of each patient from peripheral blood lymphocytes using Gene Catcher gDNA 96 × 10 mL Automated Blood kit (Invitrogen, Life TechnologiesTM, Carlsbad, CA, USA). The CHH gene panel was designed using Illumina Design Studio (San Diego, CA, USA) and included the following CHH candidate genes: *ANOS1, FGFR1, PROKR2, PROK2, GNRHR, GNRH1, GNRH2, KISS1, KISS1R, TAC3, TACR3, HS6ST1, FGF8, CHD7, DUSP6, FEZF1, FGF17, FLTR3, IL17, SEMA3A, SEMA3E, SEMA7A, SOX2, SOX10, SPRY4, WDR11, HESX1, NELF*. The 28 CHH genes, consistently represented in all sequence capture panels, were assessed for the purposes of this study. Libraries were prepared using Illumina Nextera Rapid Capture Custom Enrichment kits according to the manufacturer's protocols. All regions not correctly sequenced were recovered with NexteraVR DNA Library Preparation kit (Illumina, San Diego, CA, USA). For subsequent analyses, we included as "rare variants" (RVs) all known pathogenic, rare non-synonymous or splicing-site variants (Minor Allele Frequency, MAF ≤ 0.01) and novel non-synonymous or splicing-site variants. The frequency and the functional annotation of the identified variants were checked in public and licensed databases (Ensembl, UCSC Genome browser, 1000 Genome project, ExAC Browser, NCBI, HGMD professional), considering the ethnic groups (Europeans). We excluded common non-synonymous variants with Minor Allele Frequency (MAF) >0.01, synonymous, intronic, and 5′ or 3′ UTR variants. Each variant found was confirmed by Sanger direct sequencing using BigDyeVR Terminator v.3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) on a 3100 DNA Analyzer from Applied Biosystems (Foster City, CA, USA). In order to check for pathogenicity prediction, VarSome database (19) was used (up to October 2022): only the RVs classified as likely pathogenic, pathogenic, or variants of uncertain significance (VUS), according to the American College of Medical Genetics (ACMG) classification guidelines (20), were considered for further analysis.

Statistical methods

Statistical analysis was performed using SPSS statistical package, version 27.0 (SPSS Inc., Chicago, IL, USA). Genetic and phenotypic variables were compared between the male and female populations. Moreover, comparisons were made according to the diagnosis (either KS or nCHH), the presence of any "red flag" at clinical presentation, and the enrichment in rare genetic variants at genetic investigation. Finally, in the female population comparisons were made according to Tanner stages. Either χ^2 or Fischer's exact test was used to compare categorical variables between groups. Comparisons for continuous variables were performed using independent samples t tests (for parametric data) and independent samples Mann-Whitney test or Kruskal Wallis test (for nonparametric data). Data are expressed as mean ± SE unless otherwise indicated. A p-value <0.05 was considered statistically significant.

Results

Among our 338 patients there were 94 females (F) and 244 males (M), with a female to male ratio of 1:2.6; 147/338 patients (43.5%) had a diagnosis of KS and 191/338 (56.5%) of nCHH, with no significant differences in their prevalence between the two sexes.

Sex difference in genetic background

In the whole cohort we identified a total of 245 rare variants, which were classified according to the American College of Medical Genetics and Genomics and Association for Molecular Pathology guidelines (ACMG/AMP) (20): 18.78% (46/245) resulted to be benign, 18.78% (46/245) likely benign, 24.49% (60/245) variants of uncertain significance, 24.49% (60/245) likely pathogenic and 13.47% (33/245) pathogenic (Supplementary Tables 1, 2). Benign and likely benign variants were excluded from further statistical analysis.

We found that 36.09% (122/338) of patients harbored potentially pathogenic rare genetic variants (RVs), with no significant differences between sexes (F 35,11% vs M 36.48%). RVs were monogenic and monoallelic in 27.51% (93/338) of patients (F 10/94, 20.21% vs M 74/244, 30.33%), monogenic and biallelic (RVs in homozygosis) in 3.25% (11/338) (F 5/94, 5,32% vs M 6/244, 2,46%) and finally, there was an oligogenicity in 5.33% (18/338) of cases (F 9/94, 9,57% vs M 9/244, 3,69%). The

genetic assortment of RVs was significantly different between females and males (Figure 2), with oligogenic and biallelic variants found more frequently in females (P = 0.034). This difference is maintained even after excluding patients with *ANOS1* RVs (P = 0.036).

The prevalence of rare variants in each candidate gene is shown in Figure 3 and no significant differences were found between males and females; however, RVs within *ANOS1* were found only in males, as expected.

Sex difference in clinical presentation

Family history of pubertal delay or hypogonadism was found in 104/332 patients (31.3%): 35/92 (38.04%) females and 69/240 (28.75%) males (P = 0.246). No statistically significant differences were found in the prevalence of family history according to neither the diagnosis (KS vs nCHH) nor the enrichment in rare CHH genetic variants.

Patients with at least one "red flag", as in reproductive or non-reproductive phenotypic features associated with CHH (without considering the presence of anosmia), were 146/331 (48.33%). Such features were present in 136/238 (57.14%) male patients compared to only 10/93 (10.75%) females (P = <0.001). This prevalence was significantly higher in patients with KS (73/144, 50.69%) than in those with nCHH (73/187, 39.04%) (P = 0.022), and in those harboring rare genetic variants (64/117, 54.70%) compared to patients with wild-type gene sequences (82/214, 38.32%) (P = 0.005). When considering only the non-reproductive phenotypic features, excluding cryptorchidism and microphallus (signs of absent mini-puberty that occur only in males), the prevalence of "red flags" was still significantly higher (P = 0.01) in males (53/228, 23.24%) than in females (10/93, 10.75%) (overall 63/331; 19.0%). Also in this case, the prevalence of "red flags" was higher in patients with KS (43/144, 22.1%) than in those with nCHH (20/187, 10.7%) (P = <0.001) but only a trend toward a statistically significant difference was found in patients with an enrichment in rare variants compared to those with wild-type gene sequences (P = 0.057). The prevalence of "red flag" features associated with CHH, split into male and female population, is reported in Figure 4. The prevalence of microphallus in males was 52/203 (25.6%) and the prevalence of history of cryptorchidism was 99/231 (42.9%), with no significant difference according to diagnosis or presence of rare variants, even though there was a trend toward a greater enrichment in RVs (P = 0.05; P = 0.095 respectively). The prevalence of single non-reproductive characteristics was also evaluated. Deafness was present in 12/328 (3.71%) subjects, with no significant differences according to sex. Kidney abnormalities were present in 8/328 (2.43%) subjects and were found only in males, with a significantly higher prevalence in patients with KS than in those with nCHH (P = 0.023); among patients with kidney abnormalities, those harboring RVs were 5/8 and they all involved *ANOS1*. Midline defects were present in 31/336 (9.45%) subjects, with a significantly higher prevalence in male patients (P = 0.010) and KS patients (P = 0.007); among patients with midline defects and harboring RVs, 3/12 RVs were in *ANOS1* and 5/12 were in *FGFR1* (fibroblast growth factor receptor 1). The higher male prevalence of midline defects is maintained even after excluding patients with RVs within *ANOS1*. Bimanual synkinesis were present in 21/328 (6.40%) subjects, with no significant differences according to sex, but with a significantly higher prevalence in patients with KS than in those with nCHH (P = 0.007). The prevalence of each of the non-reproductive features associated with CHH, split into male and female populations, is reported in Figure 5.

BMI SDS was -0.04 ± 1.09 in females and 0.69 ± 1.51 in males (Figure 6), with a statistically significant difference between groups (P = <0.001). The age at diagnosis was 17.13 ± 2.82 for females and 16.75 ± 3.22 for males, with no statistically significant differences between groups (P = 0.06). A lower age at diagnosis was found in subjects with "red flags" (P = 0.01). No significant differences were found between BMI SDS and age at diagnosis in subjects carrying rare variants in candidate genes compared with those who did not.

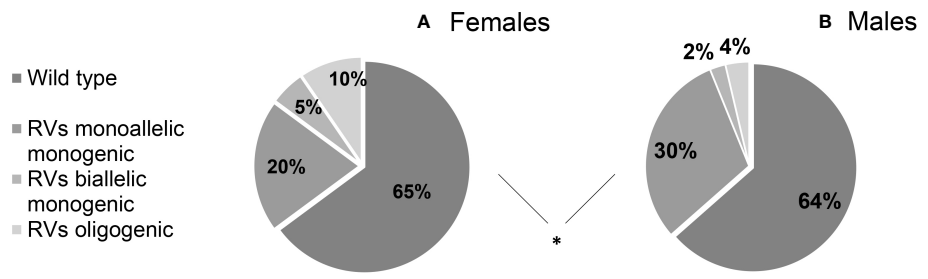

FIGURE 2
Prevalence of rare genetic variants in female **(A)** and male **(B)** cohort of patients according to their genetic assortment. RVs, rare genetic variants. Comparison between male and female patients using Fisher's exact test: *P = <0.05.

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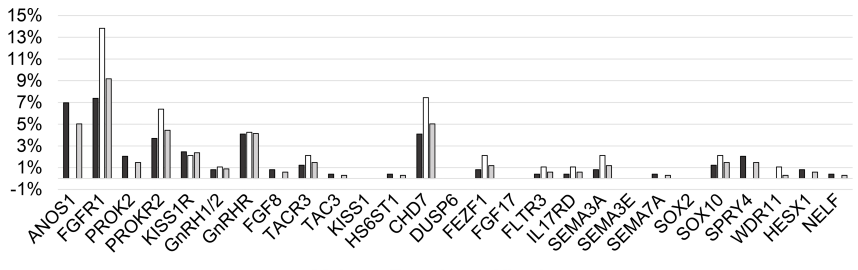

	ANOS1	FGFR1	PROK2	PROKR2	KISS1R	GnRH1/2	GnRHR	FGF8	TACR3	TAC3	KISS1	HS6ST1	CHD7
M (n=244)	17 (7,0%)	18 (7,4%)	5 (2,1%)	9 (3,7%)	6 (2,5%)	2 (0,8%)	10 (4,1%)	2 (0,8%)	3 (1,2%)	1 (0,4%)	0 (0,0%)	1 (0,4%)	10 (4,1%)
F (n=94)	0 (0,0%)	13 (13,8%)	0 (0,0%)	6 (6,4%)	2 (2,2%)	1 (1,1%)	4 (4,3%)	0 (0,0%)	2 (2,2%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	7 (7,4%)
All (n=338)	17 (5,3%)	31 (9,2%)	5 (1,4%)	15 (4,4%)	8 (2,4%)	3 (1,0%)	14 (4,1%)	2 (0,6%)	5 (1,5%)	1 (0,3%)	0 (0,0%)	1 (0,0%)	17 (5,0%)

	DUSP6	FEZF1	FGF17	FLTR3	IL17RD	SEMA3A	SEMA3E	SEMA7A	SOX2	SOX10	SPRY4	WDR11	HESX1	NELF
M (n=244)	0 (0,0%)	2 (0,8%)	0 (0,0%)	1 (0,4%)	1 (0,4%)	2 (0,8%)	0 (0,0%)	1 (0,4%)	0 (0,0%)	3 (1,2%)	5 (2,1%)	0 (0,0%)	2 (0,8%)	1 (0,4%)
F (n=94)	0 (0,0%)	2 (2,1%)	0 (0,0%)	1 (1,1%)	1 (1,1%)	2 (2,1%)	0 (0,0%)	0 (0,0%)	0 (0,0%)	2 (2,1%)	0 (0,0%)	1 (1,1%)	0 (0,0%)	0 (0,0%)
All (n=338)	0 (0,0%)	4 (1,2%)	0 (0,0%)	2 (0,6%)	2 (0,6)%	4 (1,2%)	0 (0,0%)	1 (0,3%)	0 (0,0%)	5 (1,5%)	5 (1,5%)	1 (0,3%)	2 (0,6%)	1 (0,3%)

FIGURE 3
Prevalence of rare genetic variants in each candidate gene. RVs, rare genetic variants.

Female genotype and phenotype

Anthropometric and hormonal parameters of the female cohort are shown in Table 1. The distribution of BMI we observed was comparable with that of the general population (21). No statistically significant differences were found in either hormonal or anthropometric parameters between KS and nCHH patients. Likewise, these parameters were no different between patients either harboring rare variants or not. Finally, patients with clinical "red flags" (deafness, kidney abnormalities, midline defects and bimanual synkinesis) showed only a shorter uterine length ($P = 0.018$) compared to the others. Distribution for Tanner stages is shown in Figure 7. Breast Tanner Stage and Pubic hair Tanner Stage are significantly correlated ($P = <0.001$). No statistically significant difference was found in Tanner stages at diagnosis according to either the presence of red flags at diagnosis or the enrichment in RVs. Breast Tanner Stage had a significant positive correlation with LH serum level ($P = 0.04$), ΔLH ($P = 0.01$), LH peak at LHRH stimulation test ($P = <0.0001$) and FSH serum level ($P = <0.001$) to linear regressions. However, when we compared hormonal values according to Breast Tanner Stage using non-parametric group comparison,

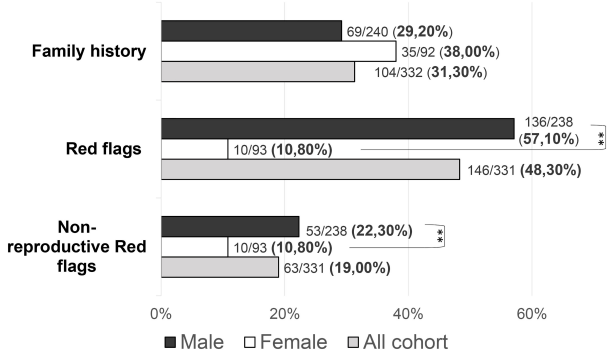

FIGURE 4
Prevalence of "red flags" and each "non-reproductive" defects in the study cohort Red flags: cryptorchidism, microphallus, deafness, kidney abnormalities, midline defects and bimanual synkinesis; Non-reproductive red flags: deafness, kidney abnormalities, midline defects and bimanual synkinesis. Comparison between male and female patients using Fisher's exact test: **$P = <0.01$.

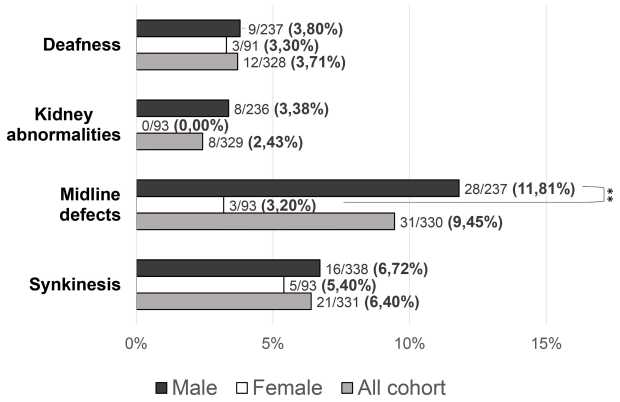

FIGURE 5
Prevalence of "non-reproductive" defects in the study cohort. Comparison between male and female patients using Fisher's exact test: **P = <0.01.

the differences in both basal and stimulated LH did not result to be statistically significant, but we still found a significant difference in basal FSH values (P = 0.036) and 17βE_2 (P = 0.041). The hormonal values according to Breast Tanner Stage are shown in Figure 8.

Discussion

In our cohort of patients with CHH we found a female to male ratio of 1:2.6. This is broadly in line with the most recent studies (11–14) and family cases sub-analyses (10), but at the same time it is different from what was described in older studies (7–10).

By using a custom NGS panel of 28 candidate genes, the prevalence of RVs as well as the rate of oligogenicity are consistent with the current literature. We found that 36.09% of patients harbored potentially pathogenic rare genetic variants, 35,11% among females and 36.48% among males respectively: the prevalence of RVs between the two sexes was not significantly different, with no evidence of a different contribution in the genetic background on the development of

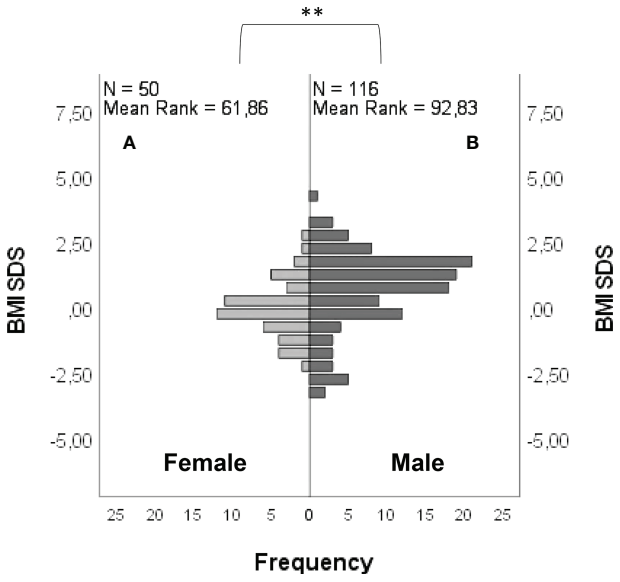

FIGURE 6
Frequency distribution of BMI SDS in female **(A)** and male **(B)** patients. Comparison between male and female cohorts using t-test: **P = <0.01.

TABLE 1 Anthropometric and hormonal parameters of female patients.

	n	Range (min; max)	Mean ± SD
		Anthropometric parameters	
Age (year)	88	10.00; 32.00	17.13 ± 2.82
Height (cm)	52	130.20; 177.00	158.81 ± 9.11
Height SDS	50	-2.88; 2.13	-0.53 ± 1.06
Weight (kg)	53	29.30; 88.50	54.35 ± 12.51
BMI (kg/m^2)	75	15.50; 36.40	22.46 ± 4.33
BMI SDS	50	-2.15; 2.79	-0.04 ± 1.09
		Biochemical parameters	
LH (U/L)	82	0.10; 6.50	0.57 ± 1.00
ΔLH (U/L)	57	0.00; 25.20	4.12 ± 5.17
LH peak (U/L)	57	0.00; 27.00	4.28 ± 5.07
FSH (U/L)	83	0.10; 7.20	1.50 ± 1.70
ΔFSH (U/L)	58	0.10; 29.00	4.76 ± 4.60
FSH peak (U/L)	55	0.39; 14.03	5.31 ± 3.62
17βE$_2$ (pg/mL)	79	4.90; 87.00	11.78 ± 14.50
		Imaging	
Uterine length (mm)	41	10.00; 64.00	35.67 ± 10.83

SDS, Standard Deviation Score; BMI, body mass index; LH, Luteinizing Hormone; ΔLH delta between basal LH and LH peak on stimulation testing. FSH, Follicle-Stimulating Hormone. ΔFSH delta between basal FSH and FSH peak on stimulation testing. 17βE$_2$ Estradiol.

the disease. In furtherance, our cohort analysis managed to reveal a difference in the genetic assortment of RVs by sex, whereby females with CHH exhibit oligogenic or biallelic variants more frequently than their male counterparts (even excluding *ANOS1*). On the other hand, there were no significant differences in the enrichment in individual variants (except for those within the X-linked gene *ANOS1*, limited to males only) although this result could also be due to the limited numerosity. The interpretation of these results is not simple, but they might hint for a stronger offsetting at the GnRH neurons level in females, such that more than one variant, or more destructive variants, are needed in order for the phenotype to occur. Still, it is recognized that the neuroendocrine reproductive axis differs between sexes in several remarkable ways, including its earlier activation in females at the time of pubertal maturation, the presence of neural circuitry that generates preovulatory hormone surges in females but not males, and the display of various sexually dimorphic reproductive behaviors (22). In particular, sex differences in the organization of kisspeptin neurons were described in rodents as the consequence of early perinatal actions of sex hormones (22–26); consistently, kisspeptin-immunoreactive neurons in humans were visualized within the preoptic region (which is involved in the positive feedback of sex steroids leading to the pre-ovulatory LH surge) only in females (24), who also seem to have a greater number of kisspeptin neurons within the arcuate nucleus (in which these cells drive the pulsatile GnRH secretion) compared to men (22, 25). However, many of the aspects of sex dimorphism in GnRH network are yet to be elucidated and may contribute to the variable expression of this disease. Another possible explanation for this sex difference is that female subjects who come to specialistic medical attention could be those with a more severe phenotype, due to a greater difficulty in recognising the CHH diagnosis, thus more likely harboring a more disrupting genetic assortment.

Moreover, several disparities between the two sexes were detected in the clinical presentation. First, we found an important difference in the prevalence of the so-called "red flags". In particular, as previously reported in literature (9, 12), kidney malformations were found only in male patients, and they were associated exclusively with RVs in *ANOS1*. In addition, midline defects in our cohort were significantly more common among males rather than females: such defects are typical of patients harboring deleterious variants within *FGF8* and *FGFR1* (27), while we also noticed an enrichment in rare variants within *ANOS1* and *FGFR1* among these patients. However, the higher prevalence of midline defects is maintained even after excluding patients harboring *ANOS1* RVs from the analysis: hence, this difference does not seem to depend on a wider involvement of an X-linked gene among males. Thus, we propose the possibility that the aforementioned sexual dimorphism within the GnRH network and – consequently – in the expression of RVs in CHH-associated genes could explain these phenotypical differences.

When we compared the SDS BMI between our female and male CHH patients, the former resulted significantly lower. In particular, while the distribution of BMI (namely SDS BMI) in our female cohort resulted broadly in line for age and sex with the reference population, males tended toward a higher BMI (21). Indeed, it is well known that body weight influences

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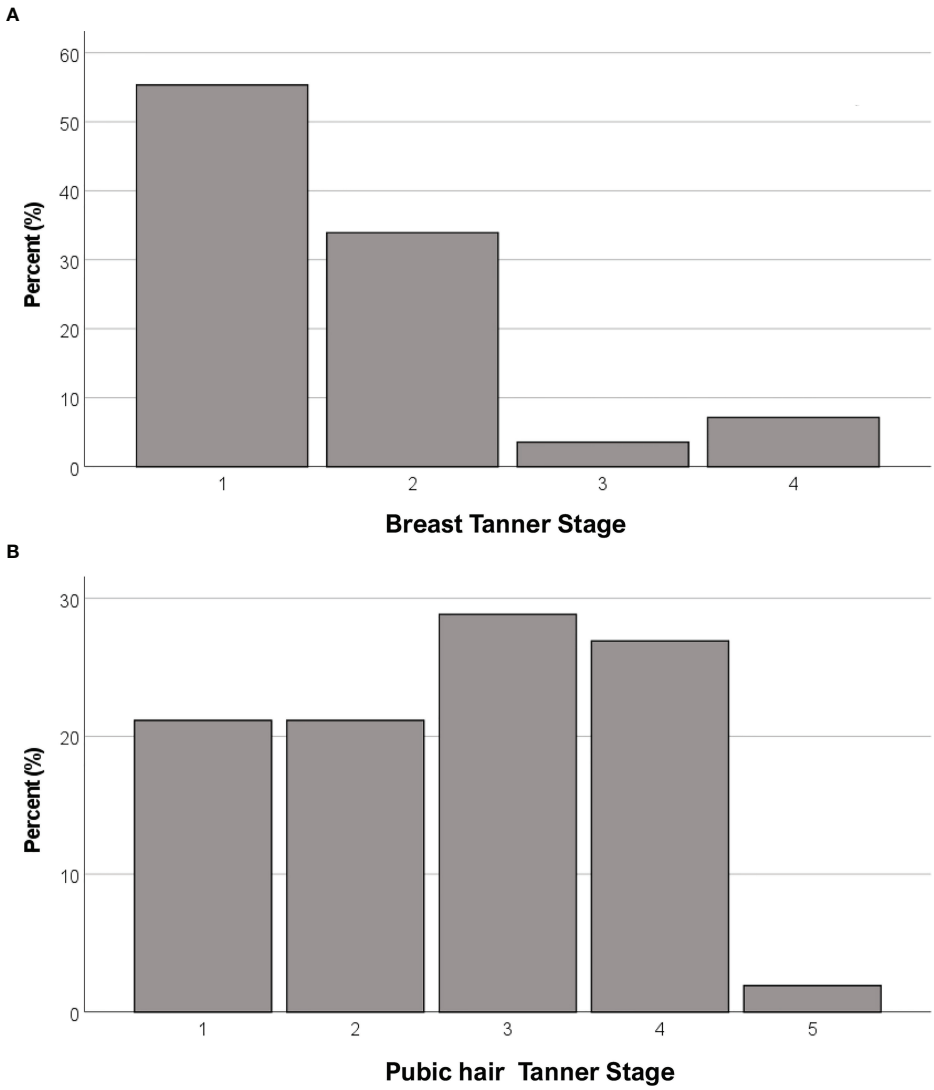

FIGURE 7
Distribution of female CHH patients for Breast Tanner Stages **(A)** and Pubic hair Tanner Stage **(B)**.

pubertal onset and reproductive function. It is established that an excessive weight in females determines pubertal advancement, while an energy deficiency can cause a functional amenorrhea; on the other hand, pubertal onset in males could be influenced by the degree of weight gain, with an earlier maturation in overweight subjects and a delayed maturation in obese ones (28). In a population of obese males with adult-onset hypogonadism it has also been pointed out that a mild form of GnRH deficiency can be characterized by a genetic origin that frequently overlaps with that of severe CHH, and obesity could be only one of the acquired cofactors involved in the onset of hypogonadism among adult subjects that are naturally prone to develop a central failure of the gonadal axis (29). Our findings are in line with the idea that patients' phenotype is the result of a complex interaction between genetic factors and metabolic/behavioral factors, with the latter having a variable influence according to sex.

Patients with CHH often have a delayed diagnosis, and such delay is particularly remarkable in females, considering the earlier physiological timing of their pubertal onset compared to boys. On the one hand, the wider diagnostic delay in girls could be explained in the light of the higher prevalence of "red flags" in males, which may facilitate the diagnostic process and therefore lead to an earlier recognition of this condition. On the other hand, this might be also justified by an insufficient awareness of this disease among gynecologists or general pediatricians that more frequently deal with primary or functional hypothalamic amenorrhea.

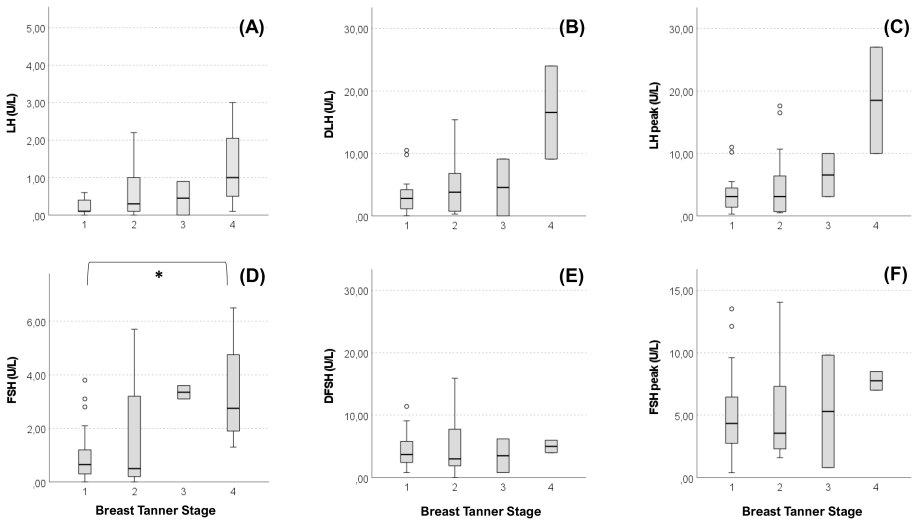

FIGURE 8
Basal and stimulated values of gonadotropins according Breast Tanner Stage. Value of basal LH **(A)**, difference between basal LH and LH peak (DLH) after stimulation test **(B)**, LH peak after stimulation test **(C)**, basal FSH **(D)**, difference between basal FSH and FSH peak (DFSH) after stimulation test **(E)**, FSH peak after stimulation test **(F)** compared according to Breast Tanner Stage. Pairwise comparison: *P = <0.05.

Regarding clinical presentation in female patients, in contrast to previous reports (30), no differences emerged between female patients with KS and nCHH. In addition, no relevant biochemical and anthropometric differences were found based on the presence of "red flags", aside for a shorter uterine length associated with non-reproductive characteristics. More than 50% of our patients were completely pre-pubertal at the time of diagnosis, but in the other cases a variable degree of pubertal advancement was observed before its arrest. From a biochemical point of view this is mainly revealed by a more pronounced LH response to dynamic testing, as observed in cases of spontaneous pubertal onset, although basal LH values remain inappropriately reduced. As can be noted from Figure 8, while a predominant FSH response over LH is maintained in patients with Breast Tanner Stage 1, which is typical of a prepubertal condition, in patients with spontaneous pubertal onset and subsequent arrest this ratio is inverted, which is an occurrence compatible with a previous activation of the HPG axis. Basal FSH values, on the other hand, seem to correlate better with pubertal advancement. However, these observations corroborate the concept of CHH as a disease with a broad clinical scenario, suggesting a possible reappraisal of those clinical forms considered spurious in the past.

The robustness of these results is constrained mainly by the limited sample size, and from the potential bias that data come from a single Centre. However, in consideration of the rarity of this disease, ours is one of the largest series ever studied with such a wide panel of candidate genes: we therefore believe that this study may still have some relevance in the current state of knowledge. Moreover, it must be acknowledged that despite our efforts in providing an accurate pathogenicity classification of the identified rare variants according to the available guidelines, the attribution of an etiologic significance to each variant is very challenging, especially in the context of oligogenicity: in this case, in fact, even rare variants theoretically considered insufficient to cause the disease by themselves could instead contribute additively in affecting the clinical phenotype.

In conclusion, despite the analysis of this large CHH cohort does not clearly point out striking differences in genetic background according to sex, intriguingly it unveils a greater prevalence of oligogenicity in females. In addition, a greater prevalence of non-reproductive phenotypic characteristics and a higher BMI emerge in male patients compared to females. These findings identify some distinctions in the clinical presentation between the two sexes that could indicate a variable expression of genetic rare variants and a dimorphic modulation of the phenotype according to metabolic/behavioral factors, which will need to be substantiated and investigated by further studies. On the other hand, we can assume that many of the epidemiological differences observed between males and females might depend on the lack of specialist diagnostic attention in a proportion of girls with delayed puberty, which could constitute a *selection bias* in cohort analysis. It is likely that the refinement of diagnostic sensitivity in recent years might explain the decrease of such gap between sexes which was observed among the latest studies.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://zenodo.org/record/6600520#.YpZ4Y1RBzb0, Zenodo database.

Ethics statement

The studies involving human participants were reviewed and approved by Istituto Auxologico Italiano ethics committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

SF, BC, MB contributed to the conception, data analysis and first draft of the manuscript. MB, LP, BC, SF, GG, LG performed patient follow-up, clinical diagnosis, and treatment. MB, SF, DM, EM, MA collected clinical data. MB, LP, VV, FH were responsible for genetic analysis and its interpretation MB, LP, BC, VV contributed to the supervision and critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.965074/full#supplementary-material

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Emotional and autonomic response to visual erotic stimulation in patients with functional hypothalamic amenorrhea

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Introduction: Functional hypothalamic amenorrhea (FHA) is a clinical condition associated with high levels of physiological and psychological stress ranging from weight loss to maladaptive behavior and coping skills. A reliable measure of the psychophysiological response to stress and the ability to cope with stimuli is heart rate variability (HRV). Through the sympathetic (SNS) and parasympathetic nervous system (PNS), the autonomic nervous system (ANS) promotes various changes in HRV that reflect the individual's psychophysiological response to stress. FHA patients are characterized by high levels of PNS activation during psychological load, suggesting that parasympathetic hyperactivation could be a pathology marker.

Methods: In the present study, we examine changes in HRV during observation of erotic, neutral, and disgusting images in 10 patients with FHA [(mean ± S.D.) age: 26.8 ± 5.9] and in 9 controls (age: 25.4 ± 6.4; BMI: 22.47 ± 2.97) to assess the differential activation of PNS and SNS between FHA patients and controls matched for age and without other clinical conditions.

Results: Our results showed that FHA patients had significantly higher HRV activation while observing high emotional value images and not during the observation of neutral images confirming a parasympathetic hyperactivation.

Discussion: HRV and cognitive and psychological testing, could provide new insights into understanding such a clinically understudied condition and provide further tools for clinical diagnosis and treatment.

KEYWORDS

functional hypothalamic amenorrhea (FHA), HRV variability analysis, RR variability, TAS 20, sex index, psychophysiology (all MeSH terms)

Introduction

Functional hypothalamic amenorrhea (FHA) is a condition in which an abnormality in gonadotropin-releasing hormone (GnRH) secretion leads to impairments in follicle-stimulating hormone and luteinizing hormone (1, 2). FHA is diagnosed after other causes have been ruled out, and its cause appears to be pulsatile hypothalamic gonadotropin-releasing hormone (GnRH) dysfunction with consequences for follicle stimulating hormone (FSH), luteinizing hormone (LH), and estradiol levels. FHA accounts for 30% of cases of secondary amenorrhea (3). Psychologically, FHA is associated with high levels of stress, excessive physical activity, maladaptive eating disorders, and weight loss (4). In recent years, many studies have focused their attention on the relationship between FHA and psychological variables that influence coping strategies in FHA patients (5). There is ample evidence that stress affects endocrine networks, in particular overactivity of the hypothalamic-pituitary-adrenal (HPA) axis has been observed in women with FHA, leading to elevated cortisol levels (6). The relationship between FHA and psychological factors is circular: psychological factors can lead to FHA and at the same time FHA has a significant impact on women's psychological well-being (7). A reliable measure of the psychophysiological response to stress and the ability to cope with stimuli is heart rate variability (HRV) (8). Changes in heart rate are an indicator of the adaptation of the cardiovascular and nervous system to the environmental requests (9). HRV is defined as the variation of the heartbeats in the time interval and is measured considering the variation in the beat to beat interval (10). A reliable index of HRV is the standard deviation between beat intervals (SDNN) calculated by excluding technical and physiological artifacts. SDNN is calculated by the square root of the total variance in the ECG recording. Low SDNN was found in patients diagnosed with PTSD, low SDNN reflect lower activity of the PNS and a reduced physiological response to cope with stress (11–13).

Another index used to characterize HRV is the root mean square of the successive difference (RMSSD), calculated as the proportion of NN intervals larger than a given threshold (14). RMSSD can be used to estimate the vagal contribution in HRV (15). RMSSD was associated with higher levels of anxiety and depression (16).The two main branches of the autonomic nervous system (ANS), the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS) influence HRV. Through the SNS and PNS, the ANS promotes various changes in HRV that reflect the individual's psychophysiological response to stress. In particular, retraction of the PNS causes activation of the SNS, resulting in the so-called fight or flight response, which is an automatic physiological response to stressful events (17). During the stress response, the hypothalamic-pituitary axis (HPA) triggers endocrine changes such as the release of corticotropin hormone from the hypothalamus (18). Several findings suggest that an adequate level of PNS activation is a protective factor in the development of mental illness (19). It was observed that individuals with high resting state HRV were characterized by less perceived stress and a high capacity for emotional self-regulation than individuals with lower resting state HRV (13, 20). High resting state HRV is associated with a predominant role of PNS over SNS. By vagal nerve activation the PNS decrease heart rate (21). The action of PNS is fast and given the short times range used in HRV measures HRV represents the activation or withdrawal of PNS (21–23). To assess the response of the ANS to stress, it is possible to assess HRV reactivity by comparing baseline HRV to HRV indices when faced with an emotionally charged stimulus. In this sense, high HRV reactivity may reflect maladaptive activation of the PNS, making subjects less responsive to the stressor (24), resulting in a delayed response. It was found that FHA patients showed increased HRV reactivity in response to stress stimulation than controls, FHA patients were characterized by increased parasympathetic activation without the typical concomitant change in heart rate due to sympathetic activation in response to orthostatic stress (25).

Galetta and colleagues (2003) (26) found a change in HRV in a sample of patients with anorexia nervosa characterized by hyperactivation of the parasympathetic nervous system and suggested that HRV and diastolic analysis are useful metrics to assess the severity of the pathology. Similar results were found by Bomba and colleagues in 2014 (5), where an HRV comparison between FHA and AN patients showed similar patterns with increased parasympathetic nervous system activation during a

24-hour recording. When subjected to cognitive load during the Stroop color word test, FHA patients exhibit low heart rate, low systolic and diastolic blood pressure, indicating hyper vagal tone that does not permit task completion, resulting in poorer performance than controls (27). However, it remains unclear whether HRV and HRV-reactivity can be a psychophysiological marker for FHA. It should be noted that FHA patients are characterized by an atypical vagal response and consequent SNS activation than the general population (25, 28), which may indicate neurophysiological and psychophysiological correlates of the disease useful in daily clinical practice.

FHA patients can be also affected by sexual dysfunction caused by hypoestrogenism causing impairment in genital receptivity and reduction in libido (29–31). However due to the complex interaction between mood disorders and hormonal dysfunction to date it is still unclear whether sexual dysfunction in FHA is due to hormonal imbalance or to maladaptive psychological behavior and coping strategies (31). To date the relationship between sexual dysfunction, FHA, neuroendocrine and psychological functioning remain unclear and no study investigated the role of physiological activation related to sexual arousal in patients with FHA.

In the present study, we examine changes in HRV during observation of erotic, neutral, and disgusting images in patients with FHA to assess the differential activation of PNS and SNS between FHA patients and controls.

Methods

Sample

A total of 19 women took part in the experiment. Ten participants were patients with FHA [(mean ± S.D.) age: 26.8 ± 5.9; BMI: 21.23 ± 2.55] and the other 9 participants were healthy subjects (age: 25.4 ± 6.4; BMI: 22.47 ± 2.97) with no history of amenorrhea or other significant clinical conditions. All participants declared to be heterosexual. All participants had no history of neuropsychiatric disorders and had normal or near-normal visual acuity. All participants were non-smokers or light smokers with a daily consumption of less than 25 cigarettes (Table 1). No subject reported drug or alcohol abuse. The study was approved by the Institutional Ethics Committee (3415/2018) and was conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent to participate in this study.

Protocol

Each experimental session started with 5 minutes cardiac baseline recording where the subject was in seated resting state position.

Participants were asked to closely watch a slideshow that featured erotic images, neutral images, or disgusting images. The

TABLE 1 Demographic, anthropometric and consumption habits of the experimental sample.

Participant	Age	Weight (Kg.)	Height (cm.)	Physical activity (min/day)	Smoker	Cigarettes consumption (Cigarettes/day)	Coffee consumption (N. coffee/day)	Alcohol consumption (units/week)
FHA patient 1	31	50	160	90	no	0	2	1.5
FHA patient 2	24	51	163	240	no	0	2	0
FHA patient 3	30	62	157	90	no	0	5	0
FHA patient 4	24	58	160	240	no	0	1	4.5
FHA patient 5	30	60	152	180	no	0	3	0
FHA patient 6	40	51	164	150	no	0	1	1.5
FHA patient 7	23	57	168	0	no	0	0	3
FHA patient 8	21	55	170	300	No	0	1	1.5
FHA patient 9	23	58	166	300	No	0	1	1.5
FHA patient 10	22	51	158	300	no	0	1	0
CONTROL 1	26	75	165	0	no	0	0	0
CONTROL 2	22	66	170	300	si	4	2	3
CONTROL 3	20	52	163	150	no	0	2	4.5
CONTROL 4	21	55	163	350	no	0	0	3
CONTROL 5	20	57	160	180	no	0	4	3
CONTROL 6	25	68	180	240	si	2	3	4.5
CONTROL 7	40	70	160	90	no	0	3	3
CONTROL 8	30	50	155	300	si	4	2	4.5
CONTROL 9	25	55	165	90	no	0	1	3

slide show was divided into three blocks, each block consisted of images of the same category (e.g. erotic, neutral, disgusting), and the order of the blocks was randomly chosen between the participants. A two-minute blank screen separated the image blocks. During this time, participants were asked to rate the images they had just seen. Rating was performed according to the IAPS database rating system by compiling VAS scales of how satisfied vs. dissatisfied, calm vs. excited, or controlled vs. in control based on the images just viewed. For each category, 24 images were selected from the IAPS database (32). Each image stayed on screen for about 5 seconds and was repeated twice in one block. The order of the blocks was randomized between participants. (Figure 1). After the cardiac recording was completed, participants completed a neuropsychological assessments composed by MoCA Test: a screening assessment for detecting cognitive impairment (24); Stroop Color Word Test (33) to assess the ability to inhibit cognitive interference; a computer-based Go-No-Go test to assess sustained attention and response control; Simple Reaction Times to assess the functioning of global attention were performed. After completing the neuropsychological assessment phase, the TAS-20 (34) and Hendrick Sexual Attitude Scale (35) were administered to assess the presence of marked alexithymia or sexual attitudes that might explain cardiac response to the specific stimuli presented.

ECG data

Electrocardiogram (ECG) data were collected with Pulse, developed by STMicroelectronics and manufactured by MR&D (Italy). Pulse is a wearable device with a sampling rate of 256 Hz. The device was attached to the center of the participant's chest with an elastic band containing electrodes. The center of the chest corresponds to lead 1 of the standard 12-lead ECG. The ECG was recorded in one session divided into 5 blocks, with the first block consisting of the baseline recording of the subjects' resting state for 5 minutes. The second, third, and fourth blocks were registered while the subject watched the slide show, each block of the slide show lasting about 5 minutes. Each slideshow block was separated by a two-minute blank slide. The last block was the recovery phase recording and lasted 5 minutes. The pulse sensor filtered the signal with a bandpass filter (0.05 to 40 Hz). The raw ECG signal saved in European Data File format (EDF+) was then transferred to Kubios HRV software 3.1 (36) for HRV analysis. A QRS detector algorithm was used to extract the beat-to-beat RR intervals from the ECG data. The ECG was also examined visually to detect and correct for artifacts such as missing or extra beats. All ECG recordings with an artifact rate of less than 5% were included in the data analysis. Very low frequency components (<0.04 Hz) were removed in a pre-processing process using a detrending approach based on smoothness priors (36, 37).

We focused on mean RR interval: the mean interval, measured in ms, between RR peaks (Figure 2), LnHFP: the logarithm of the fast Fourier transform of high frequency power; SDDN: Mean of the standard deviations of all NN intervals for each segment of the recording; RMSSD. The root mean square of successive differences between normal heartbeats (RMSSD).

Statistical analysis

All analyzes were performed with SPSS 26.0 (IBM, 2019). The normality of the data was assessed using the Shapiro-Wilk normality test, the data were normally distributed, therefore the data were analyzed using a parametric test. Within-subject differences in HRV variables recorded while subjects viewed different image blocks were compared to baseline using paired-sample t-tests. To better examine the differences between the groups, indices of difference in HRV variables were also calculated by subtracting the HRV values recorded at baseline from the HRV values recorded during the experimental blocks, and then compared the differences in cardiac indices between groups using one-way ANOVAs.

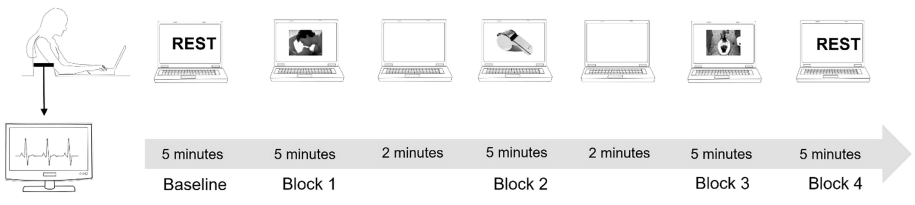

FIGURE 1
Experimental procedure.

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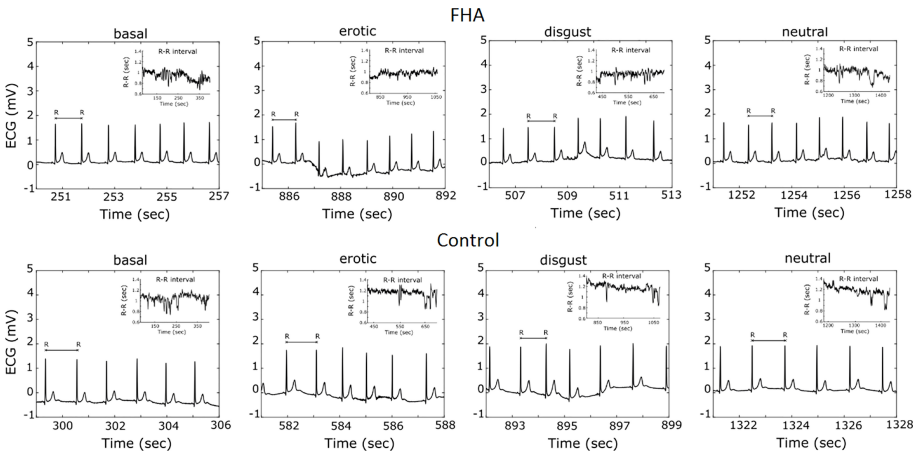

FIGURE 2
Example of Electrocardiogram recording and R-R- interval extraction in a casually selected FHA patient and Control subject.

One-way ANOVAs were also used to assess differences in psychological and neuropsychological data between groups. For all analyses, a p-value of 0.05 was considered significant.

Results

ECG data

No significant differences were found between FHA patients and controls in baseline ECG data ($p>0.05$). Comparing HRV difference indices between FHA and control group, we found significant differences in LnHFP during erotic block [(mean ± standard deviation) FHA vs Controls: 0.41 ± 0.68 vs -0.35 ± 0.66); $F_{1,17} = 6.175$ $p = 0.02$], in neutral block [FHA vs Controls, 0.28 ± 0.54 vs -0.38 ± 0.79; $F_{1,17} = 4.528$, $p = 0.04$)] and in disgust block [FHA vs controls: 0.28 ± 0.35 vs -0.27 ± 0.59; $F_{1,17} = 6.919$, $p = 0.01$] (Figure 3A). In addition, ANOVA showed differences in RMSSD in disgust block between the FHA and control groups [FHA vs Controls: 5.73 ± 5.48 vs -1.31 ± 5.66; $F_{1,17} = 7.622$, $p = 0.01$] (Figure 3B). No significant differences between groups were found in other HRV variables indexes calculated (all $p > 0.05$). Paired sample t-tests showed that mean heart rate decreased in the FHA group during observation of erotic images [baseline vs erotic, 74.88 ± 15.86 vs. 72.38 ± 16.89; $t = 3.115$ $p= 0.01$] and disgusting images compared to baseline [baseline vs. disgust:74.88 ± 15.86 vs. 71.95 ± 13.17; $t= 2.807$ $p=0.02$] (Figure 4A). The mean RR increased in the FHA group during observation of erotic images [baseline vs. erotic: 829.24 ± 150.83 vs. 862.47 ± 164.25; $t = -3.540$ $p = 0.01$] and during observation of disgusting images [baseline vs. disgust: 829.24 ± 150.83 vs. 856.19 ± 137.65; $t =-3.606$ $p = 0.01$] compared to baseline (Figure 4B). RMSSD also increased in the FHA group during observation of erotic images [baseline vs. erotic: 38.80 ± 16.73 vs 42.13 ± 16.97; $t = -2.829$ $p = 0.02$] and during observation of disgusting images [baseline vs. disgust:38.80 ± 16.73 vs 44.53 ± 19.26; $t = -3.305$ $p = 0.01$] compared to baseline. LnHFP increased in the FHA group compared to baseline only during observation of disgusting images [baseline vs. disgust: 6.13 ± 0.86 vs 6.41 ± 0.73; $t = -2.533$ $p = 0.03$]. In addition, we found that SDNN decreased in the control group during observation of erotic images compared to baseline (baseline vs erotic 61.50 ± 21.38 vs 49.49 ± 17.09; $t = 3.493$ $p = 0.01$). No significant differences were found analyzing other cardiac data in control group (all $p > 0.05$).

Neutral images had no effect on the ECG data. No significant differences were found between patient and control group in the explicit rating of images for any of the image categories presented (all $p > 0.05$) (Table 2).

Neuropsychological and psychological assessment

We calculated error interference effects in the Stroop color word test using the formula {Error Interference Sheet - [(Error Reading Sheet + Error Naming Sheet)/2]} (25) and then compared the Error Interference Index between FHA and control group. Compared to controls FHA patients showed a higher error-interference index than controls in Stroop's test [(mean ± S.D) Controls vs FHA: 0.00 ± 0.00 vs 0.30 ± 0.42); $F_{1,17}$Rosalyn= 4.530 $p = 0.048$].

10.3389/fendo.2022.982845

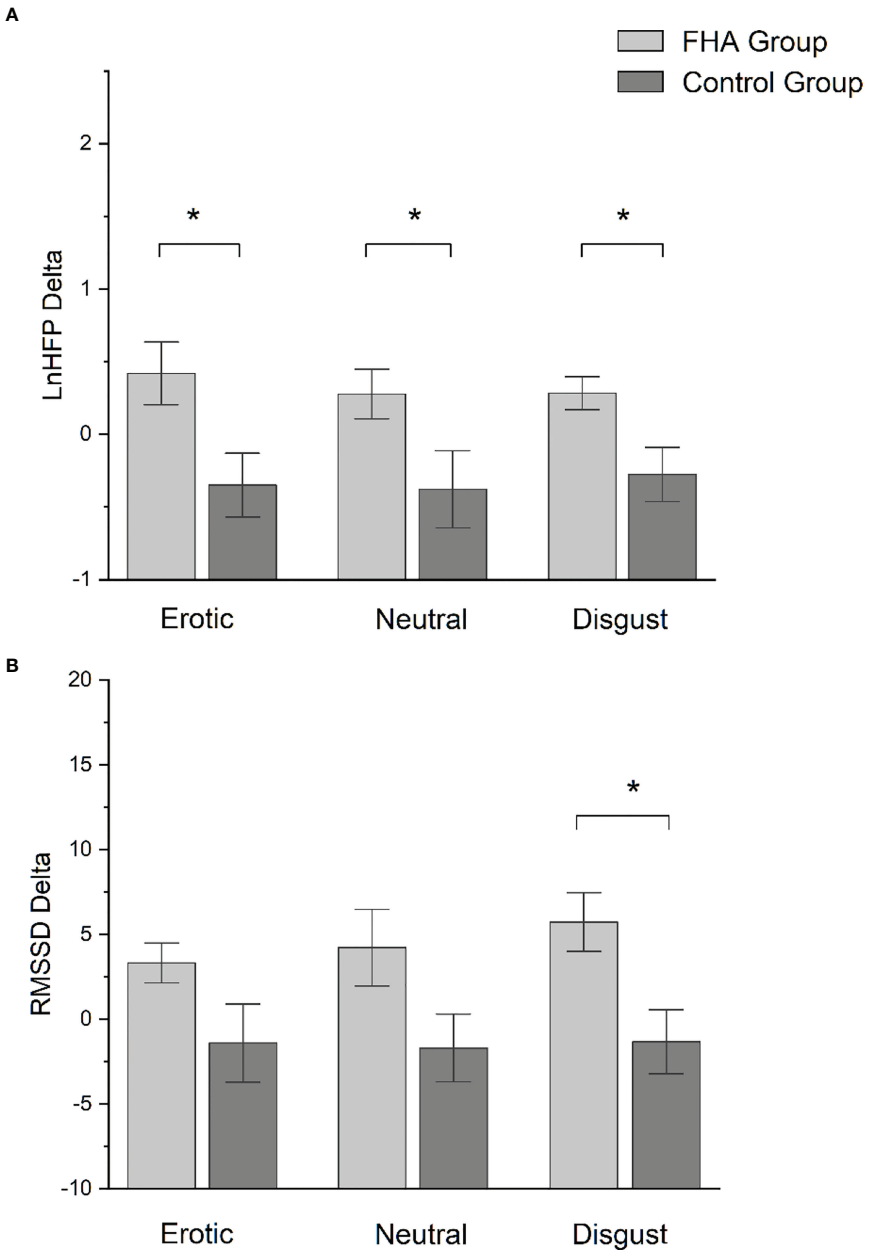

FIGURE 3
(A) Bar-chart representing lnHFP delta in FHA group and control group. Error bars represent standard errors. *p value < 0.05; **(B)** Bar-chart representing RMSSD delta in FHA group and control group. Error bars represent standard errors. *p value < 0.05.

FHA patients made more errors than controls on the go-no-go task (Controls vs FHA: 0.22 ± 0.44 vs 1.30 ± 1.25; $F_{1,17} = 5.975$ $p = 0.026$] (Table 3).

No significant differences in simple reaction times were found between groups (all ps > 0.05).

No significant difference was found in the TAS-20 scores, Hendrick Sexual Attitude Scales, between the FHA and control groups (all ps > 0.05).

Discussion

This study investigated autonomic responses to the observation of arousing pictures in a sample of patients with FHA and in controls. Our results show that FHA patients have significantly higher HRV activation during observing of high emotional value images (erotic and disgusting images) compared to baseline and not during observing of neutral images.

10.3389/fendo.2022.982845

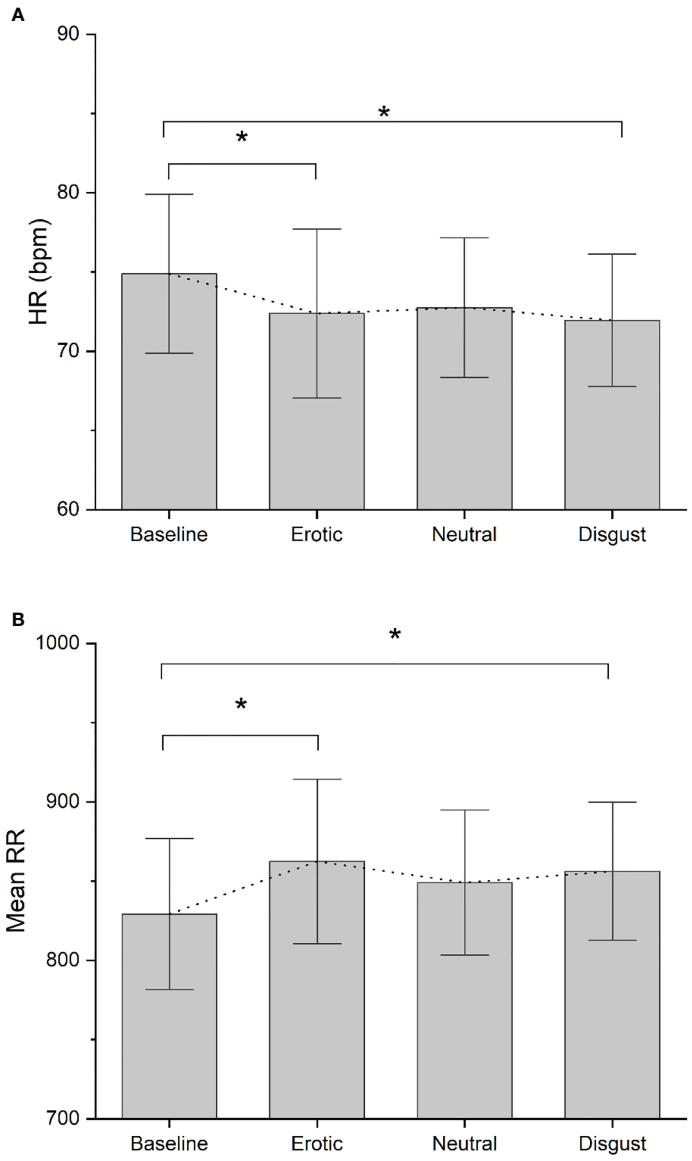

FIGURE 4
(A) Bar-chart representing Mean HR recorded in each experimental block in FHA group. Error bars represent standard errors. Dot-line connects the mean value recorded in each block. *p value < 0.05; **(B)** Bar-chart representing Mean RR recorded in each experimental block in FHA group. Error bars represent standard errors. Dot-line connects the mean value recorded in each block. *p value < 0.05.

To the best of our knowledge this is the first study assessing the HRV response to the observation of emotional pictures in FHA patients.

High HRV is associated with activation of PNS (38), in our sample it seems that FHA patients have higher PNS activation than controls during observation of images characterized by emotional valence.

While baseline HRV did not differ between FHA patients and controls, FHA patients showed higher HRV reactivity (39). HRV reactivity reflects parasympathetic activation in response to external events (19). While resting HRV has been described as adaptive, reflecting functional autonomic regulation, high HRV reactivity may reflect maladaptive activation of the parasympathetic nervous system (38, 40). Indeed, increased parasympathetic nervous system activation might reflect decreased responsiveness with delayed response mobilization to meet environmental demands (41). During challenging stimulation, a subject with a decrease in HRV may be more responsive and tend to respond with marked activation of the sympathetic nervous system to face the external stimuli (41).

Changes in HRV indices during exposure of IAPS images were studied by Kwang-Ho Choi and colleagues (42) who found

TABLE 2 Cardiological data in Functional Hypothalamic Amenorrhea group (FHA) and control group (control) for each picture block.

	Baseline		Erotic pictures		Neutral Pictures		Disgusting Pictures	
	FHA	CONTROL	FHA	CONTROL	FHA	CONTROL	FHA	CONTROL
Mean HR	74,88 ± 15,86	76,23 ± 8,25	72,38 ± 16,89	73,39 ± 10,31	72,75 ± 13,92	73,99 ± 10,51	71,95 ± 13,17	74,10 ± 10,50
Mean RR	829,24 ± 150,83	796,80 ± 102,13	862,47 ± 164,25	835,12 ± 141,78	849,06 ± 144,66	829,03 ± 145,04	856,19 ± 137,65	827,70 ± 144,30
RMSSD	38,80 ± 16,73	35,86 ± 12,96	42,13 ± 16,97	34,45 ± 15,72	43,03 ± 18,38	34,16 ± 12,55	44,53 ± 19,26	34,53 ± 14,13
LnHFP	6,13 ± 0,86	6,10 ± 0,79	6,55 ± 0,74	5,75 ± 0,91	6,40 ± 1,03	5,72 ± 0,88	6,41 ± 0,73	5,82 ± 0,49
SDNN	62,04 ± 16,68	61,50 ± 21,38	54,38 ± 15,07	49,49 ± 17,09	59,05 ± 18,70	59,86 ± 20,26	61,62 ± 14,13	52,95 ± 18,71

Mean heart rate (Mean HR); Mean Interval measured between RR peaks (Mean RR); Root mean square of successive differences between normal heartbeats (RMSSD); logarithm of the Fast Fourier Transform of the High Frequency Power (LnHFP); Mean of the standard deviations of all the NN intervals for each segment of the recording (SDNN). Data are expressed ad mean ± Standard Deviation.

a positive correlation between HRV and valence and a negative correlation between HRV and dominance. However, Kwang-Ho Choi and colleagues found HRV variations only in response to images characterized by a negative valence with a strong activation value. In our sample, controls showed no parasympathetic activation during observation of both negative and positive-scored images, while FHA patients showed higher HRV reactivity to both positive and negative-scored images. HRV reactivity in FHA patients could represent hyperactivation of the parasympathetic nervous system that needs further investigation. In addition, studies have found PNS activation during sexual arousal or disgust responses (43, 44), suggesting a possibly lower threshold for PNS activations than controls due to PNS hypertonicity in FHA patients.

Differences between FHA and controls in HRV could be due to the effects of hormone levels on cardiac activity due to parasympathetic activation (45). Leptin perfusion in the arcuate nucleus of the hypothalamus in rats was found to increase sympathetic nervous system activity (46). It should be possible that in FHA, low levels of leptin affect the neurons of the arcuate nucleus, resulting in decreased sympathetic nervous system activity and increased parasympathetic activity.

In addition, GnRH levels might affect the preoptic hypothalamus and arcuate nucleus, which play a central role in PNS activity (46–48). The preoptic hippocampus in ewes has been found to be sensitive to GnRH and that GnRH levels are elevated during the follicular phase of the oestrus cycle. FHA is associated with low calorie intake and less available energy for the organism is (49), parasympathetic hypertension leading to heart rate slowdown and bradycardia could be a protective mechanism to adapt to starvation and reduce energy expenditure (50, 51). A disease associated with low energy expenditure that overlaps with FHA is anorexia nervosa, Galetta and colleagues (2003) (26) found an alteration in HRV in a sample of patients with anorexia nervosa characterized by hyperactivation of the parasympathetic nervous system during a 24h recording and suggested that HRV and diastolic analysis might provide a useful measure for assessing the severity of pathology.

Similar results to those of Galetta and colleagues (26) were found by Bomba and colleagues in 2014 (52) where HRV comparison between FHA and AN patients showed similar patterns during a 24h recording with increased activation of the parasympathetic nervous system. Taking these results together, it is possible that FHA patients exhibit altered parasympathetic activation that could reflect a possible continuum between these two pathologies, with hormonal and psychological dysfunctions of the patients (5) together with the energy available to the organism playing a modulating role. Like anorexia nervosa, FHA is a multifaceted disease characterized by a complex interaction between psychological and physiological factors. FHA patients show similar psychopathological patterns

TABLE 3 Results of Stroop Color Word Test, GO-NO-GO Test, Simple Reaction Times, Toronto Alexithymia Scale (TAS-20), Hendrick Sexual Attitude Scales in FHA (Functional Hypothalamic Amenorrhea group) and in controls (control group).

	STROOP CW Test		GO-NO-GO		SIMPLE REACTION TIMES	TAS-20	Hendrick Sexual Attitude Scales			
	Errors	Time	Errors	Time			Permissiveness	Practice	Communion	Instrumentality
FHA	0,5 ± 0,70	27,55 ± 8,85	1,3 ± 1,25	472,75 ± 122,22	321,48 ± 42,42	43,55 ± 14,70	3,60 ± 0,55	1,56 ± 0,48	2,16 ± 0,58	3,22 ± 0,60
CONTROL	0,11 ± 0,33	24,97 ± 2,80	0,22 ± 0,44	479,25 ± 52,01	314,38 ± 18,90	52,88 ± 9,49	3,23 ± 0,36	1,47 ± 0,46	1,85 ± 0,41	3,24 ± 0,77

Data are expressed as mean ± Standard Deviation.

as anorexia nervosa patients, but at lower levels that do not meet the criteria for a clinical diagnosis (53). Differences between HRV in FHA and controls could be due to complex interactions between hormone levels, available energy and ability to cope with excitatory stimuli and everyday stressors (5). The results of the psychological questionnaire administered to our participants showed no significant differences in terms of depression, alexithymia and sexual attitudes. Our results are consistent with studies describing that FHA patients do not meet the criteria for a psychopathological diagnosis (4, 5). However, more research is needed to better examine psychological variables that may influence such findings, both in measuring personality traits and in recording clinical interviews and psychological history.

Regarding cognitive performance, we found that FHA patients made more errors and showed stronger interference effects on the Stroop CW test than controls. In the Stroop CW, subjects are asked to suppress the automatic reading of the presented words and to name the ink color in which the words are written (33). The Stroop CW test is a challenging test and is associated with increased systolic blood pressure and heart rate (54, 55) in response to cognitive load and mental stress (27). Similar to our results, Gallinelli and colleagues found that FHA patients had lower values on the Stroop CW test along with lower blood pressure and slower heart rate during the test (27). The Stroop CW test is physiologically linked to the anterior cingulate cortex (56), which is heavily involved in attentional processes. Furthermore, the anterior cingulate cortex is connected to the hypothalamus and may play a role in activating the hypothalamic-pituitary-adrenal axis (55, 56) when one is under high cognitive load or a stressful state (55). The influence of the anterior cingulate cortex on the hypothalamic-pituitary-adrenal axis may explain the effects of cognitive load and stress on ANS activation (57). However, regarding our results the link between PNS activation and performance at the Stroop CW test remain at a speculative level only since we did not recorded cardiological data during the execution of the Stroop CW Test.

Similar to the results of the Stroop color word test, we found that FHA patients made more errors on the Go-No-Go task, a cognitive-behavioral task that assesses the ability to inhibit responsive behavior (58). In rats performing a Go-NO-Go task with food reward, it was observed that increased activity in orexin neurons in the medial hippocampus was associated with their greater accuracy (59). In addition, orexin has been associated with food cravings, sympathetic activation and effects on the hypothalamic-pituitary-gonadal axis in *in vitro* and *in vivo* studies (60, 61).In humans, orexin is implicated in appetite, behavior, and psychophysical activity *via* regulation of reproductive and stress hormone secretion (62). Orexin has been found to affect eating behavior and stress response in anorexia nervosa patients, affect the hypothalamo-pituitary-adrenal (HPA) and hypothalamo-pituitary-gonadal axes, and activate the sympathetic nervous system in anorexia nervosa patients (62). Despite these preliminary results, future studies should investigate the role of orexin in FHA to better explain the complex neurophysiological and neuropsychological phenomena associated with the disease. To date, little is known about the relationship between HRV and FHA, and no study has focused on HRV response to external stimulation with emotionally fluctuating images in FHA patients. Our study presents certain limitation, statistical power could have been affected by the limited sample size, furthermore we did not have information about hormonal and metabolic profile of the sample, these data could be useful to better characterize differences between FHA patients and control subjects.

Despite the mentioned limitations, the results of our study suggest that FHA patients may have a different cardiac response than healthy controls in response to emotionally activating stimulation. Further studies are needed to clarify the role of emotional external stimulation and cognitive load in ANS activation in FHA patients. Our results suggest that it is possible to use images from the IAPS as an excitatory, reliable stimulation method to elicit HRV changes and assess parasympathetic activation. FHA is a complex disease with neuroendocrine and psychophysiological correlates. HRV, along with cognitive and psychological testing, could provide new insights into understanding such a clinically understudied condition and provide further tools for clinical diagnosis and treatment. Considering the link between FHA, mental illness (63) and the neuroendocrine (43) and psychophysiological (4, 52) features of FHA, clinical interventions should be characterized by multidisciplinary approaches. Studies on psychotherapeutic interventions for stress reduction and development of adaptive coping skills have shown promising results with cognitive behavioral therapy and hypnosis (64, 65), it might be useful to implement approaches with biofeedback based on HRV.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by San Paolo Hospital review board. The patients/participants provided their written informed consent to participate in this study.

Author contributions

NM, design of the work, collected the data, performed the analysis, and wrote the paper. AB, RosF, BP, VS, NT, and EG, contributed data or analysis tools, performed the analysis, and revised the paper. VG and AM, collected the data and revised the paper. AP and RobF, design of the work, contributed data analysis, wrote, and revised the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Psychological factors in functional hypothalamic amenorrhea: A systematic review and meta-analysis

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Background: Psychological factors have been found to be associated with functional hypothalamic amenorrhea (FHA); however, their role in the onset or persistence of FHA is still understudied. The study aims to assess the associations of psychological factors with the presence vs the absence of FHA.

Methods: A systematic literature search has been conducted across the major databases (PubMed, PsycINFO, Scopus, and Embase) to explore the psychological factors associated with FHA. The search was limited to English-written articles published from 2000 onwards. Articles were selected based on stringent inclusion/exclusion criteria. After data extraction, meta-analysis and meta-synthesis were conducted.

Results: Of 349 retrieved articles, eight studies were included. Findings indicate that the main psychological factors associated to FHA seem to be depression and eating attitudes, especially drive for thinness. FHA women present higher levels of anxiety, sleep disorders, dysfunctional attitudes, and alexithymia. The meta-analysis on drive for thinness revealed that the pooled MD across the studies was statistically significant both in the fixed 0.63 (95% CI: 0.31–0.95) and random model 0.70 (95% CI: 0.13–1.26). Likewise, as for depression, the pooled MD across the studies was statistically significant both in the fixed 0.60 (95% CI: 0.36–0.84) and random model 0.61 (95% CI: 0.20–1.01).

Discussion: Findings showed the association of psychological factors and FHA and recognized their involvement in the persistence of the disorder. A multidisciplinary approach should involve a collaborative process between gynecologists, clinical psychologists, and psychiatrists, from diagnosis to treatment. Longitudinal studies should be implemented with a comparison/control group or by including clinical psychologists in the psychological assessment and study design.

KEYWORDS

functional hypothalamic amenorrhea, amenorrhea, psychological factors, depression, eating attitudes, meta-analysis, systematic review

1 Introduction

Functional hypothalamic amenorrhea (FHA) is a form of secondary amenorrhea caused by hypogonadotropic hypogonadism related to an aberration of the pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus (1). FHA entails a significant impact on ovarian function with hypoestrogenism and the sequent absence of a regular menstrual period persisting for more than 3-6 months in women who previously had regular cycles (2, 3).

According to epidemiological data, in Europe and USA FHA accounts for 20-35% of cases of secondary amenorrhea, rising to be one of the most common reproductive disorders in women of childbearing age (4). Among adolescent girls, the prevalence of FHA is approximately 15-48% of secondary amenorrhea diagnoses (5), but that may be underestimated due to the difficulty in differentiating from the instability of hypothalamic–pituitary-ovarian (HPO) axis during puberty (6). However, once the menstrual pattern is assumed, the approach for the diagnosis of amenorrhea does not differ from that of adults (1, 6).

FHA has been found to be related to the suppression of the HPO axis; these dysregulations of the HPO axis in FHA seemed not to be caused by any identifiable organic disease or anatomic factor (1), while they were found to be associated with stress, weight loss, and/or excessive physical exercise (7). Based on these eliciting factors, three variants of FHA have been established: weight loss-related, exercise-related, and stress-related (3). As the cause of FHA does not seem organic but functional, the role of psychological factors can be decisive in assessing their impact on the onset and persistence of the disorder. In fact, a psychogenic component has been recognized in FHA since its first diagnostic formulation (8).

Considering weight loss-related FHA, evidence shows that psychological factors interact with significant physiological changes, metabolic alterations, and endocrinological aberrations, contributing to the persistence of FHA (9, 10). Indeed, women with FHA reported more deranged eating attitudes, restrictive eating behavior, and bulimic symptoms than comparison (11). As previous studies have shown, women with dysfunctional eating attitudes are at higher risk for menstrual problems and infertility (12). As it is widely known, decreased food intake causes a shortage of energy, which leads the body to economize and thus suspend those functions not necessary for survival (such as menstruation) (13). All these features are also present more severely in patients with an eating disorder, so it is essential to make an appropriate differential diagnosis.

Likewise, exercise-related amenorrhea is a frequent clinical condition among athletes, particularly those involved in elite sports and aesthetic disciplines (1), such as artistic skating and gymnastics. Several psychological and behavioral factors have been identified as contributing to the high prevalence of secondary amenorrhea in athletes. One of the main factors is high energy expenditure associated with many athletic activities, which can lead to energy deficits and disrupt regular ovulatory cycles. In addition, many athletes engage in disordered eating practices such as restricting food intake or engaging in extreme dieting to maintain leanness for optimal performance (1). Another potential factor is psychological stress associated with athletic training and overstated goals.

Concerning stress-related FHA (3), the Endocrine Society Clinical Practice Guidelines (14) claim that various types of stress and life events, which are perceived as traumatic and/or stressful experiences by women, might trigger the disease. In this sense, it has been argued that FHA is a type of somatic disorder caused by stress (15–17). Russell and colleagues (18) identified a wide spectrum of stressors that may characterize the prior experience of women with FHA, such as extreme danger, fear for one's own safety profound family conflict or loss, and minor life changes (starting school, etc.). So far, a fair number of literature reviews has been published investigating the pathophysiological factors of FHA or its consequences also in term of psychological effects (7, 19, 20), However, to the best of our knowledge, no review has been specifically designed to address psychological factors in FHA, nor their role on the onset, persistence, or subtype of FHA. The primary aim of this systematic review was to assess the associations of psychological factors with the presence (cases) vs the absence (controls) of FHA. The recognition of the involvement of such factors may help the identification of new clinical strategies for the management of FHA.

2 Materials and methods

The systematic review was conducted according to the Cochrane Collaboration guidelines (21) and the PRISMA Statement (22), to provide a comprehensive and unbiased overview of the evidence defining psychological factors associated with FHA, its onset or persistence.

2.1 Search strategy

To include the broadest range of literature, the electronic literature search was conducted on the four major databases in the field of health sciences: PubMed, PsycINFO, Scopus, and Embase.

The search was limited to articles published from 2000 onwards (in order to focus on more contemporary psychological constructs and assessments) and to English-language journal articles. The literature search was undertaken on the 24th of February 2022 and was adapted for each database as necessary. The search strategy includes Medical Subject Headings (MeSH) and text words for the following domains of interest: 1) secondary amenorrhea/secondary amenorrhoea/functional hypothalamic amenorrhea; AND 2) psychology/psychological/mental health, OR 3) stress/stressor/distress, OR 4) emotions/emotional/affective/alexithymia. The selection of the search term was based on the literature on FHA and the clinical experience. Lateral searching of Google Scholar and of the reference lists of included studies was executed manually. An expert librarian was consulted to verify that the search strategy was adequate to yield a comprehensive literature search. Figure 1 shows the process of literature search and selection of publications.

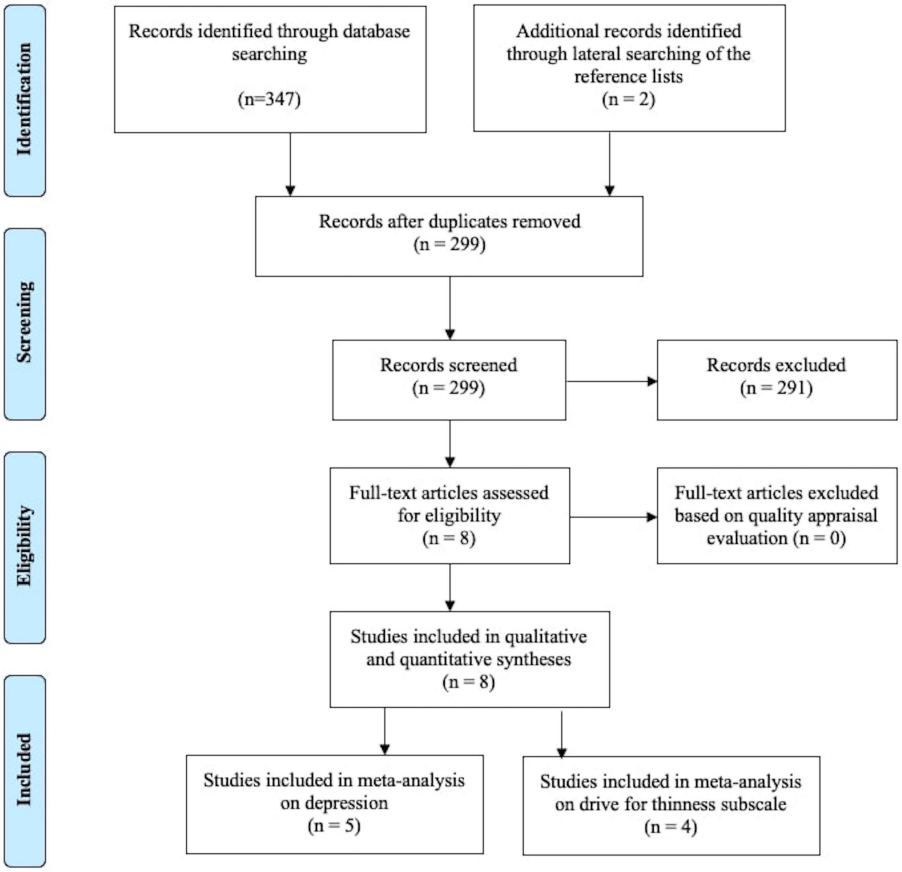

FIGURE 1
Flow diagram of literature search and process of studies' selection.

2.2 Eligibility criteria

The inclusion criteria were as follows:

- diagnosis of FHA based on current criteria (1);
- peer reviewed studies;
- studies adopting an analytical study design (i.e., an observational study with a control or comparison group);
- studies addressing psychological dimensions using standardized and validated instruments.

Exclusion criteria were:

- intervention studies;
- non-empirical studies (e.g., case reports, commentaries, reviews, abstract meeting, or letters);
- studies not addressing psychological dimensions;
- not available full text.

2.3 Study selection and data extraction

Study selection was performed independently by two researchers (FB, LB), through the screening of records (i.e., titles, abstracts, and keywords). If the abstract was insufficient to determine the eligibility of the article, screening was based on the full text. When the two researchers did not reach a consensus on inclusion, decisions were made through discussion with a third researcher (GP).

A standardized data form was prepared in Excel to simplify data management. The following information was extracted for each eligible article: title, authors, country of the first author, keywords, article type.

2.4 Quality assessment

The "Quality appraisal checklist – quantitative studies reporting correlations and associations" by the National Institute for Health and Clinical Excellence (NICE) (23) was used to assess quality of studies. Two reviewers (FB, LB) independently applied criteria to assess a study's internal and external validity and address the following key aspects of study design: characteristics of study participants; definition of independent variables; outcomes assessed, and methods of analyses. Checklist items are worded so the following responses are possible: "++" =2; "+" = 1; "-" = 0., "Not Reported (NR)" = 0. Since Items 3.4 and 3.5 are not applicable to any of the included studies, they were not considered in the total count. For each article, the sum of all scores was calculated; the highest possible score was 34. The threshold for high quality was set at 75% (minimum

score=25.5) of the total score. Disagreements were resolved through discussion with a third researcher (GP).

The quality assessment did not eliminate any studies, rather it provided a comprehensive view of the quality of the studies' methodology, analysis, and presentation of results (Supplementary Table A).

2.5 Meta-synthesis and meta-analysis

First, a meta-synthesis was undertaken to synthesize evidence and provide a comprehensive overview of the data. Thus, two reviewers (FB, LB) independently identified psychological dimensions based on psychological constructs (e.g., depression, anxiety, etc.) describing the findings that emerged from the included studies. Data were clustered and summarized to describe the identified dimensions.

The primary analysis was calculated using descriptive statistics reported in the results section of each study and involved differences in the distributions of psychological factors between FHA women and controls (or comparison). Since all studies compare mean values and standard deviations (SDs) across case/control groups on continuous outcome variables, effect sizes (expresses as mean difference) and the corresponding 95% confidence interval (CI) were performed using Cohen's d (24) or Hedge's g (25) and its 95% confidence interval (26) for each outcome measure. Specifically, Cohen's d was calculated when two groups have similar standard deviations and are of the same size; while Hedges' g was computed when there are different sample sizes. Cohen's d or Hedge's g values were interpreted as small if $0.2 \leq$ d (or g) < 0.5, medium if $0.5 \leq$ d (or g) < 0.8; large if d (or g) ≥ 0.8 (27).

Then, because the included studies were heterogeneous in terms of variables and instruments, it was not always appropriate to undertake a meta-analysis (21). A decision was made to only conduct a meta-analysis if four or more studies assessed the same psychological construct, as already made in previous studies (28).

Meta-analysis was performed using the Comprehensive Meta-Analysis Software program (26).

Statistical heterogeneity was assessed with the I^2, which has been conventionally adopted to indicate low, moderate, and high heterogeneity values of 25%, 50% and 75%, respectively (29). As heterogeneity among studies was expected on the basis of large variability in the assessment of psychological constructs across different studies, both fixed and random-effects models were used (30).

To investigate potential publication bias, the funnel plot of the results of the included studies was checked for asymmetry (31).

3 Results

3.1 Study characteristics

Of the 349 publications found, 347 were identified by database search and 2 by an additional search based on the articles' bibliography. The process of removing duplicates and screening titles and abstracts resulted in 8 publications. Therefore, we included 8 studies in the review (Table 1), of which two (36, 37) refers to the same study population (and were therefore included but jointly analyzed). All studies used a case-control design (women with FHA vs healthy controls), with 3 studies also included a comparison group (organic amenorrhea or due to anorexia nervosa -AN-), for a total of 514 women: 209 with FHA, 239 eumenorrheic controls, and 66 with other forms for amenorrhea.

3.2 Psychological factors

The psychological variables detected by the selected studies are described in Table 2.

The psychological aspects assessed are numerous, including both symptomatologic dimensions as depression, anxiety, stress, sleep disorders, or eating disorders, and more stable or trait dimensions as alexithymia, self-control, perfectionism, need for social approval, or resilience. Sexual functioning is included in one study.

3.3 Depression

Five studies (11, 32–34, 38) evaluated the role of depression in FHA using self- report instruments.

We observed heterogeneity in the populations involved and in the questionnaires adopted in each study. Three studies involved adult women (32, 33, 38) and assessed depression through the Beck Depression Inventory (32, 38) or the Zung Self-Rating Depression Scale (33); while two studies involved FHA adolescents assessing depression through the Children Depression Inventory (11, 34).

Considering depression, a significant difference in level of depression between FHA patients and controls was found both in adult (32, 33) and adolescent (34) populations (respectively: d = 0.77 [0.21 – 1.33]; d = 0.83[0.37 – 1.29]; d = 1.25 [0.59 – 1.90]). In contrast to these results, the study by Pentz and colleagues (38) did not find a significant difference between FHA women and healthy controls, neither on the scale of depression (BDI) nor with the scale of mood disorders (Profile of Mood States).

The pooled MD across the studies was statistically significant both in the fixed 0.60 (95% CI: 0.36–0.84) and random model 0.61 (95% CI: 0.20–1.01); the heterogeneity between the studies included was moderate (I^2 63%) (Figure 2).

The funnel plot (Supplementary Figure A) showed a low risk for publication bias.

Considering the comparison between FHA patients and women with other amenorrhoeic conditions, a non-significant difference was found between FHA patients and patients with organic amenorrhea (32) while a significant difference was found between FHA patients and AN patients (d = -0.99[-1.63 – -0.35]) (34), with FHA adolescents reporting less depressive symptoms when compared to adolescents with AN.

3.4 Eating disorders

Seven studies (11, 32, 34–38) evaluated the role of eating attitudes and eating disorders in FHA using self- report instruments. Specifically, four studies used the Eating Disorder Inventory-2 (EDI-2) (11, 32, 34, 38) finding differences between FHA patients and controls or comparison groups with respect to the EDI-2 subscales (see Table 2 for all data). In particular, the subscale drive

10.3389/fendo.2023.981491

TABLE 1 Characteristics of the studies included in the review.

Author (year); Country	N sample Mean age, SD	N control/ comparison (Mean age, SD)	Variables observed	Measure	FHA versus controls [Effect size (CI 95%)]	FHA versus comparisons [Effect size (CI 95%)]
Marcus et al. (32); **USA**	28 with FHA 26.4 ± 5.6	24 with organic amenorrhea 26.2 ± 4.7 25 eumenorrheic 25.9 ± 5.1	Depression	Beck Depression Inventory Hamilton Raing Scale for Depression	Higher levels of depressive symptoms both in Beck Depression Inventory [d= 0.77 (0.21-1.32)] and Hamilton Raing Scale for Depression [d= 0.74 (0.19 -1.30–)]	NS
			Disfunctional Attitude	The Disfunctional Attitude Scale	Higher dysfunctional attitudes [d= 0.83 (0.27-1.40)]. Specifically, greater need for approval [d=0.85 (0.29-1.41)]	NS
			Sefl-control	Self-Control Scale	NS	NS
			Eating attitude	Eating Disorder Inventory-2 Bulimia Test	Higher drive for thinness [d=1.17 (0.41-1.94)] Higher bulimia [d=1.12 (0.36-1.88)] Higher ineffectiveness [d = 0.77 (0.04-1.50)] Higher interoceptive awareness [d=0.90 (0.16-1.64)] Higher bulimic symptoms [d=1.42 (0.63-2.21)]	Higher drive for thinness [d =0.89 (0.20-1.59)] Higher bulimia [d=0.04 (-0.66-0.67)] Higher ineffectiveness [d=0.91 (0.21-1.61)] Higher interoceptive awareness [d==0.73 (0.05-1.42)] Higher bulimic symptoms[d==0.91 (0.22-1.61)]
Bomba et al. (11); **Italy**	20 with FHA (10 N-FHA and 10 Hy-FHA) 16.4 ± 0.9	20 eumenorrheic 17.1 ± 1.2	Depression	Children's Depression Inventory	NS	NA
			Eating attitudes	Eating Disorder Inventory-2;	N-FHA: Higher drive for thinness [g = 0.30 (-0.45-1.07)] Hy-FHA: Higher Ineffectiveness [g=0.67 (-0.11-1.45)] Higher Ascetism [g =0.71 (-0.07-1.49)] Higher Impulse regulation [g=0.26 (-0.50-1.02)] Higher Social Insecurity [g= 0.71 (-0.07-1.48)] Higher EDI-2 total score [g= 0.52 (-0.24-1.29)]	
			Previous trauma events and life events, psychosomatic disorders, and depressive and anxiety traits	Psychodinamic semistructured interview for girls and their parents	Mild subclinical depressive trait Subclinical eating disorders in adolescence Stressfull events were reported by parents of FHA patients; mainy, precence of psychoatric disease in famiy, postpartum depression, intrafamiliar conflicts, transferts, and chornic disease Reported life events associated with the onset of amenorrhea were common life events that these teenagers felt as highly stressful (such as change of school or breaking up with boyfriend). Eighty percent of FHA girls reported having an excessively demanding, controlling, and intrusive mother, with conflicting family relationships in 50% of cases.	
Dundon et al. (33); **USA**	41 with FHA 26.1 ± 5.5	39 eumenorrheic 25.1 ± 4.9	Sexual function	McCoy Female Sexuality Questionnaire	Lower scores on sexuality [d = 1.22 (0.74-1.69)]	NA
			Depression	Zung Self-Rating Depression Scale	Higher depression [d = 0.83 (0.37-1.29)]	
			Anxiety	Zung Self-Rating Anxiety Scale	Higher anxiety [d =1.26 (0.79-1.75)]	

(Continued)

10.3389/fendo.2023.981491

TABLE 1 Continued

Author (year); Country	N sample Mean age, SD	N control/ comparison (Mean age, SD)	Variables observed	Measure	FHA versus controls [Effect size (CI 95%)]	FHA versus comparisons [Effect size (CI 95%)]
Bomba et al. (34); **Italy**	21 with FHA 16.2 ± 0.9	21 with anorexia nervosa 15.9 ± 1.1 21 eumenorrheic 16.2 ± 1.1	Depression	Children's Depression Inventory;	Higher scores of depression at CDI [d =1.25 (0.59-1.90)]	Lower scores at CDI [d =-0.99 (-1.63 - -0.35)].
			Eating attitude	Eating Disorder Inventory-2	Higher drive for thinness [d=1.21 (0.55 – 1.87)] Higher maturity fears [d=1.30 (0.63-1.96)] Higher social insecurity [d = 0.94 (0.30-1.58)]	Lower drive for thinness [d= -0.78 (-1.41–0.16)] Lower ineffectiveness [d= -0.65 (-1.27- -0.03)]; Lower interpersonal distrust [d=-0.87 (-1.50 - -0.24)] Lower interoceptive awareness [d=-1.08 (-1.73 - -0.44)]
			Alexithymia	Toronto Alexthymia Scale-20	Higher scores of alexithymia [d= 1.27 (0.61-1.93)]; and the difficulties in describing feelings subscale [d = 1.38 (0.71-2-05)]	Lower scores at difficulty in identifying feelings [d= -0.94 (-1.58- -0.30)]
Pentz and Radoš (35); **Croatia**	25 with FHA 21 (18-24)[1]	21 with organic amenorrhea 23 (21 - 26)[1] 20 eumenorrheic 24 (20.5 – 26.5)[1]	Trait measurements	Multidimensional Perfectionism Scale Self-Control Scale	Higher levels of perfectionism trait [d= 1.39 (1.06-1.72)] Higher levels of concerns over mistake [d= 1.08 (0.85-1.31)]	Higher levels of personal standards [d= 0.73 (0.61-0.85)]
			Eating attitudes	Adolescent Dieting Scale + Eating Attitude Test	NS	NS
			Parental rearing perception	Memories of upbringing	NS	NS
			DSM-IV Disorders	Structured Clinical Interview for DSM-IV Disorders (SCID)	History of anorexia nervosa	History of anorexia nervosa
Tranoulis et al. (36, 37); **UK**	41 with FHA (17.8 ± 1.8)	86 eumenorrheic (18.3 ± 2.7)	Sleep disorders[a]	Athens Insomnia Scale[a]	Higher scores at the following subscales of AIS-8[a] [g= 0.55 (0.17-0.93)]: awakenings during the night [g== 0.71 (0.32-1.09)] final awakening [g=1.13 (0.73-1.52)] total sleep duration [g= 0.65 (0.27-1.03)] quality of sleep [g= 1.6 (1.18-2.02)]. Lower sleepiness during the day[a] [g=-1.0 (-1.39 - -0.61)]	NA
			Anxiety[a,b]	State-Trait-Anxiety-Inventory[a,b]	Higher scores of STAI g=1.10 (0.70 - 1.50)]	
			Eating Attitudes[a,b]	Eating Attitude Test-26[a,b]	Higher scores of EAT-26 [g= 1.36 (0.95-1.77)][a] Higher scores of EAT-26 subscales[b]: specifically dieting [g= 0.90 (0.51-1.29)], bulimia and food preoccupation [g= 1.72 (1.29-2.15)].	
			Overweight preoccupation[a,b]	Multidimensional Body-Self-Relations Questionnaire[a,b]	Higher scores of MBSRQ [g= 1.06 (0.67-1.45)],	
			Physical Activity[a,b]		Higher scores of IPAQ [g= 0.57 (0.19-0.94)].	

(Continued)

TABLE 1 Continued

Author (year); Country	N sample Mean age, SD	N control/ comparison (Mean age, SD)	Variables observed	Measure	FHA versus controls [Effect size (CI 95%)]	FHA versus comparisons [Effect size (CI 95%)]
				International Physical Activity Questionnaire[a,b]		
Strock et al. (38); USA	33 with FHA (21.2 ± 0.5)	28 eumenorrheic (24.1 ± 0.9)	Mood	Profile of Mood States	NS	NA
			Depression	Beck Depression Inventory	NS	
			Stress	Daily Stress Inventory Perceived Stress Scale	NS	
			Eating Attidues	Three-Factor Eating Questionnaire Eating Disorder Inventory	Higher drive for thinness [d= 1.00 (0.47-1.54)] Higher cognitive restraint [d= -4.16 (3.26 -5.05)]	
			Disfunctional Attitude	Dysfunctional Attitude Scale	Greater need for social approval [d= 2.7 (2.00 – 3.39)]	
			Resilience	Brief Resilient Coping	NS	

[1]Data refers to median and interquartile range.
*The appropriate Effect size measure for each variable is calculated. Specifically, Cohen's d is reported when two groups have similar standard deviations and are of the same size. While Hedges' g is calculated when there are different sample sizes.

for thinness, which assesses excessive concern with dieting, preoccupation with weight, and fear of weight gain, was found to be significantly higher in FHA patients when compared to controls or organic amenorrhoeic patients, with large effect sizes (32, 34, 38), and significantly lower when compared to patients with AN, with moderate effect sizes (34).

The meta-analysis on the subscale drive for thinness (Figure 3) revealed that the pooled MD across the studies was statistically significant both in the fixed 0.63 (95% CI: 0.31–0.95) and random model 0.70 (95% CI: 0.13–1.26); the heterogeneity between the studies included was moderate (I2 65%). The funnel plot (Supplementary Figure B) showed a low risk for publication bias.

Moreover, two studies (35–37) used the Eating Attitude Test-26, with contrary findings: while Tranoulis and colleagues (36, 37) found a significant difference in EAT-26 scores between FHA patients and controls (g= 1.36 [0.95-1.77]), and specifically higher dieting [g= 0.90 (0.51-1.29)], bulimia and food preoccupation [g= 1.72 (1.29-2.15)], the study by Pentz & Rados (35) did not found difference in disordered eating behaviors between FHA and controls; however, FHA patients reported more prevalent history of anorexia nervosa (20% vs 0% and 0%, respectively).

3.5 Other psychological symptomatologic dimensions: anxiety, stress, sleep disorders, psychiatric disorders

Two studies assessed anxiety, both in term of level of state (current) and trait (lifetime) (33, 36, 37), using two different measurement scales (Zung Self-rating Anxiety Scale and State-Trait Anxiety Scale respectively). Moreover, the study of Strock and colleagues (38) explore anxiety symptoms through a subscale of the Profile of Mood States.

A significant difference in level of anxiety between FHA patients and controls was found (33, 36, 37) (respectively: d = 1.26 [0.79 – 1.75]; g = 1.10 [0.70 – 1.50]). Higher prevalence was shown among FHA patients.

The study by Strock and colleagues (38) assessed the role of stress through two self-reported questionnaires, without showing significant difference between FHA patients and controls.

One study (36) explored the presence of sleep disorders finding significant differences between FHA patients and controls regarding sleep problems (see Table 2).

Two studies explored psychopathological dimensions by clinical interviews, one using a psychodynamic semistructured interview (11) and one using the Structured Clinical Interview for DSM-IV Disorders (SCID) (35), with miscellaneous results (see Table 2 for all details). Briefly, Bomba and colleagues (11) showed that FHA patients reported: i) an absence of important depressive and anxious disorders in FHA patients, similarly to control subjects; ii) a presence of mild subclinical depressive traits in FHA patients; iii) a presence of subclinical eating disorders in adolescence; iv) presence of previous stressful events reported by their parents and presence of common life events associated with the onset of FHA.

Also, the study by Pentz & Rados (35) revealed that the prevalence of depressive or anxiety disorders did not differ between FHA and control or comparison groups, while FHA patients reported more prevalent history of anorexia nervosa (20% vs 0% and 0%, respectively).

TABLE 2 Psychological assessment.

Topics of assessment	Questionnaire	Notes for use and studies specificities
Alexithymia	Toronto Alexithymia Scale-20 (TAS-20) (39)	Toronto Alexithymia Scale-20 (TAS-20) is a 20-item self-reported questionnaire which evaluates the presence of alexithymia. It uses a 5-point Likert scale. TAS-20 measures 3 dimensions: difficulty in identifying feelings, difficulty in describing feelings to others, and externally oriented thinking. The presence of alexithymia is defined when the score is over 61; scores from 51 to 60 are considered as at risk (39).
Anxiety	*State-trait Anxiety Inventory (STAI)* (40)	The STAI is a 20-item self-report questionnaire which assesses the levels of state and trait anxiety. It uses a 4-point Likert scale from "not at all" to "very much". The STAI consists of two separate self-report scales each containing 20 questions. The first one evaluates how the patients 'currently feel' (state anxiety). The second one assesses how they 'generally feel' (trait anxiety). The total score on both subscales ranges from 20 to 80, with higher scores indicating greater levels of anxiety. Cut-off points is 40 on either subscale for all the translations (40).
	Zung Self-Rating Anxiety Scale (41)	The Zung Self-Rating Anxiety Scale is a 20-items self-report inventory which evaluates the anxiety severity. Each of the 20 items is scored using a 4-point scale ranging from 1 to 4. The anxiety score ranges from 20 to 80. In clinical screening, the recommended cut-off is the index scores of 36. In research, the cut-off of 40 would be most appropriate. (41, 42)
Axis I mental disorders	*SCID-I-RV* (43)	SCID-I-RV is a structured clinical interview created for making DSM-IV Axis I mental disorders diagnosis. It resumes the modules related to the DSM-IV sections.
Depression	*Zung Self-Rating Depression Scale* (44)	The Zung Self-Rating Depression Scale is a 20-items self-administered survey which assesses depression symptoms. The depression score ranges from 25 to 100. For recognizing adults with depressive disorder, the recommended cut-off is index scores of 50 and over (44).
	Children's Depression Inventory (CDI) (45)	Children's Depression Inventory (CDI) is a 27 items self-report questionnaire which assesses symptoms of depression in children and adolescents. Total score ranges from 0 to 54. The scores of 19 are considered the cut-off beyond which the subject is considered characterized by depressive symptoms, while the scores of 17 and 18 detect subjects at risk (45).
	Beck Depression Inventory (46)	The Beck Depression Inventory is a 16-item self-report questionnaire that evaluates the severity of depression. Scores ≥ 10 are representative of clinically significant depressive symptoms (46).
	Beck Depression Inventory-II (47)	Beck Depression Inventory-II is a 21-item self-report questionnaire which measures the severity of depressive symptoms. The questionnaire follows the criteria for major depressive disorder following the fourth edition of the Diagnostic Statistical Manual. Cut-off scores are: scores of 14–19 for mild depression, scores of 20–28 for moderate depression, scores ≥28 for severe depression (47).
	Hamilton Rating Scale for Depression (48)	The Hamilton Rating Scale for Depression is a 17-items clinical rating scale which assesses the severity of depression. The test is based on a Likert scale of either 0 to 4 or 0 to 2. Scores can range from 0 to 54. For identifying depression, scores ≥ 14 are used as a reference (48).
	Dysfunctional Attitude Scale (49)	The Dysfunctional Attitude Scale is a 40-item self-report scale that assesses the presence of dysfunctional attitudes usually held by persons predisposed to depression. Each item consists of a 7-point Likert scale (7 = fully agree; 1 = fully disagree). The score is the sum of the 40-items with a range from 40 to 280; higher scores are representative of greater dysfunction (49).
Eating attitudes and disorders	*Bulimia Test—Revised (BULIT-R)* (50)	The Bulimia Test-Revised is a 36-item self-report questionnaire which evaluates symptoms of bulimia nervosa and binge eating. Items are presented in a 5-point Likert scale. Scores are obtained by summing responses. Scores ≥104 can be used as a cut-off to indicate diagnosable bulimia nervosa (50).
	Eating Attitude Test (EAT-26) (51).	The EAT is a 26-items self-report questionnaire which assesses symptoms of eating disorders. It includes three sub-scales: dieting (13 items), bulimia and food preoccupation (6 items), oral control (7 items). It uses a six-point scale from "never" to "always". The score ranges between 0 and 78. The presence of abnormal eating behavior is defined when the score is at or above 20 (51).
	Multidimensional Body-Self Relations Questionnaire (MBSRQ) (52)	The Multidimensional Body-Self-Relation Questionnaire is a 69-item self-report inventory which assesses self-attitudinal aspects of the body-image. Possible answers are organized on a five-point scale (from "definitely disagree" to "definitely agree"). A total score at or above 2.5 confirms the presence of Overweight Preoccupation. The MBSRQ was translated and validated in Greek population (52).
	Three Eating Factor Questionnaire (53).	The TFEQ is a 51-item self-administered questionnaire which evaluates three dimensions of eating behavior. The TFEQ consists of three subscales: cognitive restraint (0-10 low, 11-13 high, 14-21 clinical range) disinhibition, and perceived hunger. The answers are scored 0 or 1 and must be added together (53)..
	Eating Disorder Inventory-2 (EDI-2) (54)	Eating Disorder Inventory-2 is a 91 item self-report questionnaire which assesses eating attitudes usually associated with eating disorders. The 91 items are on a 6-point Likert scale, from never to always, and are divided into 11 main subscales: drive for thinness (DT), bulimia (BU), body dissatisfaction (BD), ineffectiveness (IN), perfectionism (P), interpersonal distrust (ID), interoceptive awareness (IA), maturity fear((MF), asceticism (ASC), impulse regulation (IR), social insecurity (SI). Subscale scores are obtained by adding all item scores on each subscale Each item can be score with a scoring system. It transforms scores ranging from 0 to 3 rather

(Continued)

TABLE 2 Continued

Topics of assessment	Questionnaire	Notes for use and studies specificities
		than 0 to 5: a score from 1 to 3 is considered as a "symptomatic" response (always= 3, usually=2, and often = 1), and 0 is assigned to the three "asymptomatic" responses (sometimes, rarely and never (54).
	The Adolescent Dieting Scale (ADS) (55)	The Adolescent Dieting Scale is a 8 item questionnaire that assesses three strategies of dieting: calorie counting, decrease of food intake and skipping meals. It uses a four-point scale from 0 (never) to 3 (almost always). The total score ranges from 0 to 24. A score from 1 to 6 indicates the presence of minimal dieting, a score from 7 to 14 indicates intermediate dieting, and a score of 15 or higher indicates extreme dieting (55).
Mood states	*Profile of Mood States (POMS)* (56)	The Profile of Mood States (POMS) is a 65-item self-report questionnaire which evaluates transient mood states. The POMS examines six different mood dimensions: anger-hostility, confusion-bewilderment, depression-dejection, fatigue-inertia, tension-anxiety, and vigour-activity. The presence of a good mood is confirmed by high scores in the vigour subscale and low scores in the five other subscales. A Total score is calculated by summing the totals for the negative subscales (tension, depression, fatigue, confusion, anger) and subtracting the totals for the positive subscales (vigor and esteem-related affect) (56).
Perfectionism	*Multidimensional Perfectionism Scale* (57)	The Multidimensional Perfectionism Scale is a 35-item self-report questionnaire that assesses different levels of multidimensional perfectionism. All the items are organized in six subscales: Concern over Mistakes (9 items), Personal Standards (7 items), Parental Expectations (5 items), Parental Criticism (4 items), Doubts about Actions (4 items) and Organisation (6 items). Each item is rated on a five-point Likert Scale from 1 (disagree completely) to 5 (agree completely). The total score ranges from 35 to 175: a higher score is representative of higher levels of perfectionism (57).
Physical activity	*International Physical Activity Questionnaire (IPAQ)* (58)	The International Physical Activity Questionnaire is a 27-items self-report questionnaire that measures the physical activity in adult patients aged 15 to 69 years old. There are 3 possible levels of physical activity: low, moderate, or high. The questionnaire was translated and validated in a Greek population (58, 59)..
Self-control	*Self-control Scale* (60)	The Self-Control Scale is a 36-item measure which assesses the ability to cope or learned resourcefullness. It uses a 5-point scale. Higher values indicate greater self-control (60).
Sexual function	*Italian McCoy Female Sexuality Questionnaire* (61)	MFSQ-I is a questionnaire which assesses sexual function. It is divided into two factors supported by principal component analysis: Sexuality, called MFSQ-Sex (9 items) and Partnership, called MFSQ-Partner (5 items). The sexuality factor (range of scores 9–49), includes items on sexual desire, sexual arousal, orgasm, and satisfaction or enjoyment of sexual activities. The partnership factor (range of scores 5–35), includes items about satisfaction with partner as a lover, emotional closeness achieved with partner, and feeling attractive to one's partner. In both factors, higher scores indicate higher levels of function (61).
Resilience	*Brief Resilience Scale* (62).	Brief Resilience Scale is a 6-item self-rating questionnaire that assesses the ability to recover from stress. It examinates personal characteristics like resilience, coping styles, social relationships, and health-related outcomes. The total score is obtained adding the value (1–5) of responses, creating a range from 6-30 and dividing the sum by the total number of questions answered. The presence of low resilience is defined when the score is under "3" (62).
Stress	*Daily Stress Inventory* (63)	Daily Stress Inventory (DSI) is a 58-item self-report measure that assesses the stressfulness of events occurred during the last 24-hour period. It generates three daily scores: a frequency score, an impact score, an average impact rating. They are calculated by dividing the sum of impact rating by the frequency of events (63).
	Perceived Stress Scale (64)	Perceived Stress Scale is a 14- item self-report questionnaire which assesses individual stress levels. It uses a five-point Likert scale, from "never" to "very often". Total score ranges from 0 to 56. Scores are obtained by reversing the scores on the seven positive items and then summing. (64).
Perceptions of parental rearing behaviors	*Egna Minnen av Barndoms Uppfostran; one's memories of upbringing (EMBU))* (65)	Memories of upbringing is 81 item self-report questionnaire evaluating the own memories of parental rearing behavior. Each item is rated on a four-point Likert scale from 1 (never) to 4 (always). The study of Pentz and Radoš adopted a shorter version of 23 items that is considered as a valid equivalent cross-culturally instrument. It consists of three subscales: parental Rejection (7 items), Emotional Warmth (6 items) and Overprotection (9 items). Two forms are given, one for perception of mother's behaviors and one of father's behaviors (65)..
Sleep	*Athens Insomnia Scale* (66)	The Athens Insomnia Scale is an eight-item self-report questionnaire which assesses sleep difficulty. It is rated on a 0-3 scale. A cut-off of 6 indicates the diagnosis of insomnia. The questionnaire is validated in a Greek adult population. (66)

3.6 Psychological trait dimensions: alexithymia, dysfunctional attitudes, parental rearing perception

The study by Bomba and colleagues (34) assessed alexithymia and found a significant difference between FHA adolescents and healthy controls in relation to alexithymia and one of its subscale reflecting difficulties in describing feelings [respectively d= 1.27 (0.61-1.93) and d = 1.38 (0.71-2-05)]. FHA adolescents reported higher levels of alexithymia.

Three studies assessed other dysfunctional attitudes as self-control, perfectionism, and need for social approval (32, 35, 38), finding that FHA women reported higher dysfunctional attitudes [d= 0.83 (0.27-1.40)] (32) and, specifically: a significant effects on a greater need for approval (32, 38) [respectively, d=0.85 (0.29-1.41) and d= 2.7 (2.00 – 3.39)], higher levels of perfectionism trait [d= 1.39 (1.06-1.72)]

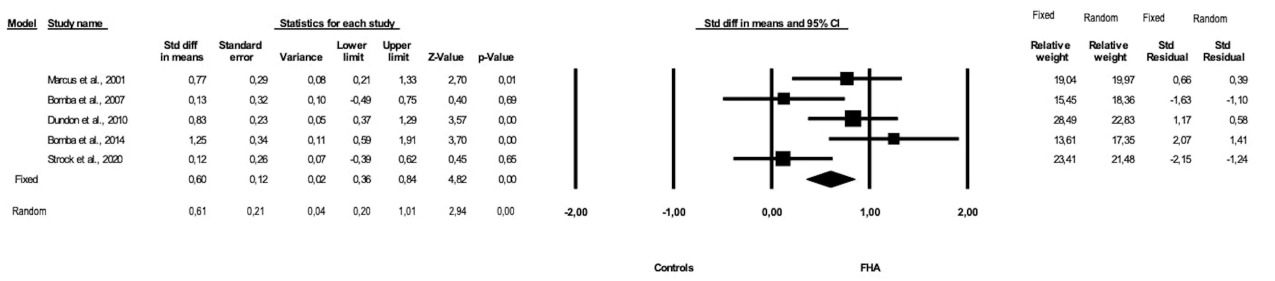

FIGURE 2
Forest plot of depression prevalence in FHA patients and controls.

and higher levels of concerns over mistake [d= 1.08 (0.85-1.31)] compared to controls (35). No difference was found regarding self-control neither compared to control (32, 38, 43) nor to comparison (32, 35) groups.

Two studies assessed the parental rearing perception, one with a questionnaire (35) and one through a clinical interview (11) with contrasting findings. While the study by Pentz & Rados (35) showed that the FHA group did not differ from controls in perception of parental rejection, emotional warmth or overprotection, the study by Bomba and colleagues (11) found that 80% of FHA girls reported having an excessively demanding, controlling, and intrusive mother, with conflicting family relationships in 50% of cases.

3.7 Sexual functioning

The study by Dundon and colleagues (33) also explore the sexual functioning of FHA patients, showing a significant difference on sexuality between FHA patients and controls [d = 1.22 (0.74-1.69)], but not on satisfaction with partner. Specifically, FHA women reported lower scores on sexuality scale that includes sexual desire, sexual arousal, orgasm, and satisfaction or enjoyment of sexual activities when compared to controls

4 Discussion

FHA is a form of secondary amenorrhea with a complex etiology, in which numerous factors have been implicated as specific triggers for its onset. In this review, we present an overview of the psychological factors associated with FHA.

All reviewed studies found that psychological factors played a role in FHA condition. Despite the heterogeneity of studies and populations, we identified some recursive psychological factors that might be clinically relevant. It is possible to divide findings in two main categories: those exploring psychological state, which might be transitory, like depression, eating attitudes, anxiety, and sleep disturbances; those focusing on psychological traits, which may represent benchmark index of patients functioning, like alexithymia, stress, and life events.

As for the symptomatologic dimensions, studies found numerous significant results on psychological state of FHA patients, identifying depression and eating attitudes as the most common psychological disturbances in FHA patients. A significant level of mood symptoms and depressive scores was showed among FHA women and adolescents (11, 34). Women with FHA experienced significantly higher depression than healthy women (33). However, two studies (11, 35) assessing psychopathological dimensions by clinical

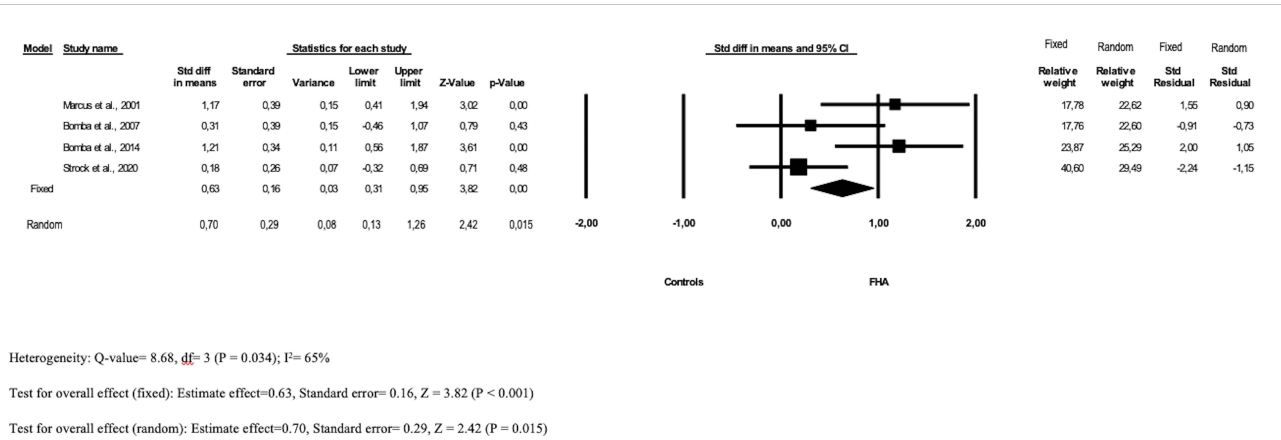

FIGURE 3
Forest plot of drive for thinness subscale prevalence in FHA patients and controls.

interviews, showed that FHA patients do not manifest important depressive disorders, with scores similar to those of control subjects. According to these findings, patients with FHA may often manifest depressive symptoms; however, they do not satisfy criteria for a clinical diagnosis for depressive disorder (11, 35).

Eating attitudes and behaviors were investigated, finding difference between FHA patients and controls/comparison groups. These findings are in line with previous studies (13, 67) showing that several dysfunctional attitudes towards nutrition, such as a focus on diet, extreme physical activity, and fear of gaining weight, characterize the psychological profile of FHA patients. Dysfunctional eating attitudes and subthresholding restrictive anorexia nervosa seem to be associated with FHA (11). Although patients with FHA do not meet the psychopathological criteria for a diagnosis of AN, they reported a higher prevalence of previous diagnosis of anorexia nervosa (35).

Overall, it is noteworthy that the symptoms that emerged *via* self-report were not confirmed by the interviews performed (11, 35). Further studies are needed to assess psychological variables with instruments other than the self-report measures, which work well as screening measures but have no diagnostic purpose.

Furthermore, reviewed studies have investigated other psychopathological symptomatology. Studies by Dundon and colleagues (33) and Tranoulis and colleagues (37) found a significant difference in anxiety levels between FHA patients and controls, while the study by Strock et al. (38) did not identify any significant findings. One study (36) explored the presence of sleep disturbances, finding significant differences with a higher prevalence among FHA patients.

One of the most popular hypotheses on the etiology of amenorrhea is that stress is one of the important pathogenetic factors in FHA patients. Although this hypothesis has been confirmed over time (6, 15–17), surprisingly only one study has investigated the role of stress in the diagnosis of FHA, revealing no significant findings. This finding is in contrast with previous literature. For example, Berga and colleagues (68) in a case-control study found that the group consisting of women with FHA had a higher concentration of cortisol than eumenorrheic women due to stress. According to the authors, high cortisol levels characterize the clinical condition of patients with FHA and, considering the recognized association between cortisol and stress, they count stress among the factors involved in the onset of FHA (68). Specifically, a key endocrinological aspect of stress is hyperactivation of the hypothalamic-pituitary-adrenal axis (6). Stress increases the secretion of corticotropin-releasing hormone and consequently contributes to the elevated levels of adrenocorticotropin and cortisol secretion, which reduce GnRH drive (7). However, it is difficult to find a clear definition of what 'stress' means in relation to FHA. Lazarus and Folkman (69) claim that stress originates from those situations in which the individual perceives his/her own resources to be insufficient to cope with the situation. To date, the definition and the nature of the stress underpinning FHA are still an intriguing matter of debate. Thus, further studies should define stress related to FHA and clarify its role.

Concerning psychological traits, reviewed studies explored alexithymia, dysfunctional attitudes, and parental rearing perception, which can represent benchmark index of patients functioning. An interesting study (34), compared alexithymia levels between FHA adolescents and control, showing that FHA patients have more difficulties in describing feelings. Alexithymia refers to the difficulties that individuals gave in perceiving, differentiating, and expressing emotions. This condition often characterizes the psychological profile of patients with somatic disorders (70) and it could be also a characteristic of women with FHA. In the reviewed studies FHA women reported higher dysfunctional attitudes such as higher level of perfectionism, higher concerns over mistakes, and greater need for approval than controls (32, 35). This is in line with previous literature (13, 14, 68), which have defined the psychological profile of women with menstrual problems, identifying low self-esteem, introversion, fear of judgement, high expectations of themselves in addition to those identified by this review.

As for parental rearing perception, studies found contrasting results (11, 35). However, in one of two studies 80% of FHA girls reported an excessive, demanding, controlling and intrusive mother, with conflict family in 50% of cases. Furthermore, stressful life events, such as presence of psychiatric disease in family, post-partum depression, intrafamiliar conflicts, transfers, and chronic disease are often reported in patients with FHA and felt very stressful, having an impact on the FHA condition (18).

Still concerning relational variables, Dundon and colleagues (33) investigated satisfaction with partner relationships and sexuality. No differences emerged in partner satisfaction, but a study (33) showed a significant difference in sexuality between FHA patients and controls. FHA women reported lower scores on the sexuality scale including sexual desire, sexual arousal, orgasm, and satisfaction or pleasure with sexual activities than controls. Although sexuality appears to be a central aspect of the psychological functioning of women with FHA, only one study has explored it (33).

Findings showed the association of psychological factors with the diagnosis of FHA and recognized their involvement in the persistence of the disorder. A multidisciplinary approach is fundamental in the clinical management of FHA and should involve a collaborative process between gynecologists, clinical psychologists, and psychiatrists, from diagnosis to treatment. The condition of FHA requires attention because it may reflect psychological or psychiatric difficulties and/or have a serious impact on daily life and quality of life. The primary intervention for women with amenorrhea is often solely focused on physiological aspects, but evidence has shown the positive impact of psychological interventions in terms of disease outcomes. Berga and colleagues (71) showed that a cognitive behavioral intervention designed to minimize problematic attitudes was likely to result in resumption of ovarian activity than observation. Based on the literature and results of this review, it is noteworthy that a tailored behavioral psychological intervention offers an effective treatment option that complements the clinical approach aimed at the recovery of ovarian activity.

The review presents some limitations. The review is based on only 8 studies; the small number of studies testifies to the fact that there is still a paucity of data regarding the psychological factors associated with FHA. Likewise, the number of patients composing the samples involved in the included studies is small. The heterogeneity of the studies' designs and instruments allowed the meta-analysis to be carried out on a limited number of variables. Moreover, different instruments have been used to measuring the same construct. Since

no study presented a prospective design from the time of diagnosis onwards, it was not possible to identify causal relationships between psychological factors and FHA and draw clear conclusions on the role of psychological factors in FHA.

Based on these considerations, further studies, possibly including epidemiological studies with prospective designs, are needed to clarify the temporal patterns and causal relationships between psychological factors and FHA. Likewise, additional studies on FHA are warranted to explore the role of psychological factors other than eating attitudes and depression. Specifically, the nature and the role of the stress underpinning FHA should be explored.

Author contributions

FB, GP, DL, EV, and LB were involved in the study conceptualization and methodology development. FB and GP performed the search. LB and FB conducted data analysis. FB, GP, and LB were involved in writing the original draft of the manuscript. EV and DL reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.981491/full#supplementary-material

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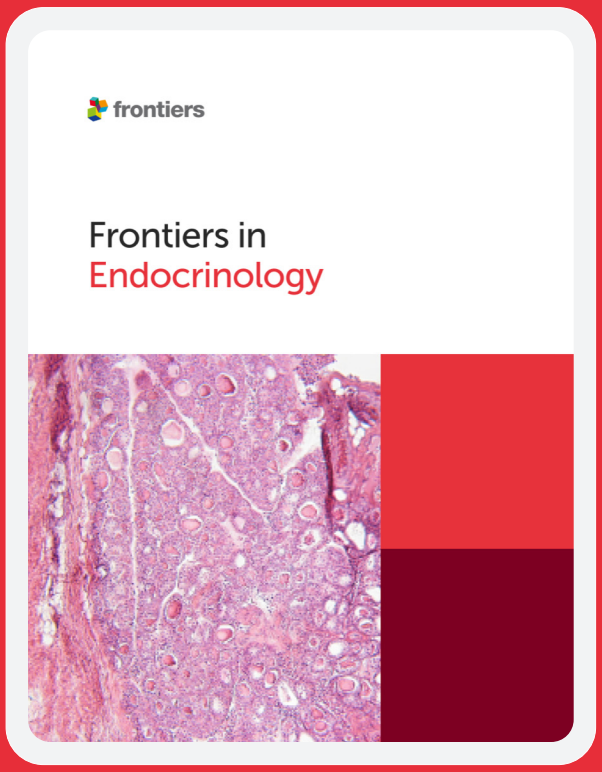

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