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Role of Hormonal alteration and FSHR genes polymorphism in development of primary amenorrhea of Upper Egyptians teenagers.

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Abstract

Amenorrhea is the absence or abnormal cessation of menses. Primary and secondary amenorrhea describes the occurrence of amenorrhea before and after menarche, respectively. We aimed to investigate the hormonal changes and FSHR gene polymorphism in teenager females with primary amenorrhea. For this purpose, 100 adult women (age 25 ± 10 years) were examined for the appearance of the signs of puberty and menstruation for detecting the cases of primary amenorrhea. The women were divided into two main groups, normal women and amenorrhea. Gonadotrophic hormones, overian hormones, anti-mullerian hormone (AMH), oxidative/ antioxidant redox and Follicular stimulating hormone receptor (FSHR) were tested in all groups. Our results showed that women with amenorrhea suffered from high FSH, LH and prolactin compared to normal women. In addition, a significant reduction of ovarian hormones was detected in women with amenorrhea compared to normal women with a concurrent alteration of antioxidant/oxidative redox. A significant reduction of antimullerian hormone (AMH) and FSH receptors (FSHR) also recorded in amenorheastic women. We can have concluded that amenorrhea is associated with sever hormonal dys-regulation, AMH could be used as a significant biomarker in diagnosis of amenorrhea and FSHR plays a pivotal role in the development of amenorrhea.

***Keywords*:** Amenorrhea, FSH, FSHR and infertility

1. Introduction

A regular menstrual cycle is a marker of physical and mental well-being in all the women of reproductive age. Multiple factors can act as disruptors of the hypothalamic–pituitary–ovarian (HPO) axis physiology and cause menstrual disorders [1].

Disruption of the hypothalamic–pituitary–ovarian (HPO) axis physiology strongly implicated in the menstrual disorders as pituitary FSH and LH are considered the main key regulator of menstruation [2]. Amenorrhea, defined by the World Health Organization (WHO) as the complete absence of bleeding or spotting for 90 consecutive days [3].

Neuroendocrine control of reproduction by brain-secreted pulses of gonadotropin-releasing hormone (GnRH) represents a longstanding puzzle about extracellular signal decoding mechanisms. GnRH regulates the pituitary gonadotropin's follicle stimulating hormone (FSH) and luteinizing hormone (LH), both of which are heterodimers specified by unique subunits (FSH/LH). Contrary to LHb, FSHb gene induction has a preference for low-frequency GnRH pulses [4]. FSH and its receptor (FSHR) are necessary for normal follicle development because FSH-deficient female mice are infertile due to a block in early (preantral) follicle development and follicle atresia [5]

FSH secreted by pituitary gonadotrophs in a cyclic manner, driven by patterned hypothalamic gonadotrophin-releasing hormone (GnRH) secretion and related neuroendocrine feedback mechanisms, plays a major role in the recruitment and development of healthy ovarian follicles through to the pre-ovulatory stage [6].

Primary amenorrhea is defined as the failure of initiation of menses by the age of 14 years in the absence of secondary sexual characteristics or at the age of 16 years with proper development of secondary sexual characteristics [7]. Primary amenorrhea can be diagnosed if a patient has normal secondary sexual

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characteristics but no menarche by 16 years of age. If a patient has no secondary sexual characteristics and no menarche, primary amenorrhea can be diagnosed as early as 14 years of age [8]**.** If a patient has normal secondary sexual characteristics, including pubic hair, the physician should perform MRI or ultrasonography to determine if a uterus is present. Müllerian agenesis (the congenital absence of a vagina and abnormal uterine development [usually rudimentary]) causes approximately 15 percent of primary amenorrhea. The most common cause of amenorrhea is hypogonadotropic hypogonadism (low FSH and LH levels) in primary amenorrhea is constitutional delay of growth and puberty [9].

Secondary amenorrhea is the absence of menses for three months in women with previously normal menstruation and for nine months in women with previous oligomenorrhea. Secondary amenorrhea is more common than primary amenorrhea [10]**.**

The most common cause of secondary amenorrhea is pregnancy. After pregnancy is ruled out, the initial work-up should be based on patient history and physical examination findings. Prolactin levels should be checked in most patients. The risk of amenorrhea is lower with subclinical hypothyroidism than with overt disease. However, the effects of subclinical hypothyroidism on menstruation and fertility are unclear, and abnormal thyroid hormone levels can affect prolactin levels; therefore, physicians should consider measuring thyroid-stimulating hormone (TSH) levels [11]**.**

Hypothalamic amenorrhea (HA) and polycystic ovary syndrome (PCOS) are the two most common causes of secondary amenorrhoea other than pregnancy [12].

Follicle stimulating hormone (FSH), secreted by pituitary gland, is one of the gonadotropins that belong to the glycoprotein hormone (GPH) family. FSH is a heterodimer consisting of one α and one β subunit, which exhibits pseudo 2-fold symmetry. FSH receptor is a number of G-protein coupled receptors and it belongs to the leucine-rich-repeat-containing Gprotein coupled receptor subfamily (LGR) which means it contains the Leucine-rich ectodomain. Like other G-protein coupled receptors, FSH receptors have seven trans-membrane helices and transduce signals to downstream molecules through the disassociated Gα d-subunit of the heterotrimeric G-protein [13].

FSH affects follicular growth, maturation, dominant follicle selection as well as estradiol production [14]. Gonadotropin levels can further help determine the source of the abnormality. Elevated FSH or LH levels suggest an ovarian abnormality (hypergonadotropic hypogonadism). Normal or low FSH or LH levels suggest a pituitary or hypothalamic abnormality (hypogonadotropic hypogonadism). Magnetic resonance imaging (MRI) of the sella turcica can rule out a pituitary tumor while normal MRI indicates a hypothalamic cause of amenorrhea **[8].**

FSH receptors (FSHRs) are necessary for normal follicle development. FSHRs are also localized with androgen receptors (ARs) on granulosa cells, are responsible for FSH-dependent granulosa cell proliferation [15]. The complex reciprocal interaction between FSH and androgen action in ovarian function is well established. FSH and LH act in the ovaries and regulate ovarian cycle in which they exert their biological functions by directly binding the extracellular domain of their specific receptors FSHR and LH/ choriogonadotrophin receptor [15].

Anti-Müllerian hormone (AMH) is a dimeric glycoprotein belonging to the transforming growth factor β (TGF-β) superfamily [16]. It has also been proven that it has a strong influence on the function of ovaries, especially on the growth of follicles [17]. AMH is expressed in endometrial and endometriotic cells [18], its measurement in serum is reflected by the secretion of AMH in the gonads. For example, in serumof women who have their ovaries removed, the AMH appears at an untraceable amount. In women, AMH is secreted by the ovarian granulosa cells of pre-antral and antral follicles starting at the 36th week of gestation [19].

Estrogen is a steroid hormone that is responsible for the growth and regulation of the female reproductive system and secondary sex characteristics. Estrogen is produced by the granulosa cells of the developing follicle and exerts negative feedback on LH production in the early part of the menstrual cycle. However, once estrogen levels reach a critical level as oocytes mature within the ovary in preparation for ovulation, estrogen begins to exert positive feedback on LH production, leading to the LH surge through its effects on GnRH pulse frequency. Estrogen also has many other effects that are important for bone health and cardiovascular health in premenopausal patients [20].

In addition to the effects on the female reproductive functions, estrogens also play a significant role in the regulation of skeletal homeostasis,lipid and carbohydrate metabolism, electrolyte balance, skin physiology, the cardiovascular system and the central nervous system [21, 22].

Prolactin is a polypeptide hormone that is synthesized in and secreted from specialized cells of the anterior pituitary gland, the lactotrophs. The hormone was given its name based on the fact that an extract of bovine pituitary gland would cause growth of the crop sac and stimulate the elaboration of crop milk in pigeons or promote lactation in rabbits [23].

Materials and methods:

100 mature female (25 ± 8 years). The average weight (50 kg ± 7 kg). There were divided into two group (n =50) according the appearance of maturation signs. Group 1: normal female where the signs of maturation regularly appeared. Group 2: no signs of maturation could be detected.

Patient's criteria

- **Inclusion criteria**

Mature females with an average age 25- 30 years, with no detected menstruation.

- **Exclusion criteria**

Adult women showed menstruation even once.

Methods:

Laboratory findings and investigations: serum FSH, LH, prolactin, estrogen and progesterone were measured by using ELISA kits (Sigma-Aldrich, Egypt). Serum GSH, TAC and MDA were measured by using colorimetric assay kits (Bio-diagnostic company for chemicals, Egypt). Serum AMH was measured by using ELISA kits (Sigma-Aldrich, Egypt).

Molecular and genetic investigations:

Genetic investigation

Venous blood samples were taken from patients and controls, blood samples was collected in EDTA vacutainer tubes. Samples were stored frozen at (- 80° c) until assay. Genomic DNA was extracted using DNA extraction kit. A single nucleotide polymorphism (SNP) genotyping and a meta-analysis of the rs6166 variant for FSHR gene was performed, by using "Real-Time PCR".

DNA extraction was performed by using Qiagen protocol and concentration purity of the end product was measured by the nanodrop instrument. Meta-analysis and genotyping of rs6166 variant for FSHR gene was performed by using real-time PCR (step1 classic real-time instrument), TaqMan Universal Master Mix II Kit.

FSHR gene polymorphism (rs6166) Polymorphism: C/T, Transition Substitution Context Sequence [VIC/FAM]:

AGGGACAAGTATGTAAGTGGAACCA[C/T]TG GTGACTCTGGGAGCTGAAGAGCA.

FSHR gene (rs6166) have two probes, one of these probes dyed by FAM dye and the other probe dyed by VIC dye, each of these probes have a different wavelength. FAM dye is C, VIC dye is T.

These probes yield Homozygous T (TT) or Homozygous C (CC) and Heterozygous (TC) according to the wavelength. If the result gives:

2 curve FAM--------- Homozygous C (mutation).

2curve VIC----------Homozygous T (Wild)

1curve VIC, 1 curve FAM -------Heterozygous TC

These are all possibilities which we are looking for in the results on which our study is based.

STATISTICAL ANALYSIS

Date entry and data analysis were done using SPSS version 24 (Statistical Package for Social Science). Data were presented as number, percentage, mean, standard error. Chi-square test and Fisher Exact test were used to compare between qualitative variables. Independent sample t-test was used to compare quantitative variables between groups. P-value considered statistically significant when $P < 0.05$.

Results

Changes in serum FSH, LH and prolactin hormones concentration:

Our results in table (1) showed a significant increase in serum FSH, LH and prolactin hormones concentration in Amenorrhea group as compared to normal females at ($P < 0.05$).

Table (1): changes of FSH, LH and prolactin:

Groups	FSH (mIU/ L)	LH (mIU/ L)	Prolactin (µg/ L)
Normal	10.15 ± 0.12	9.54 ± 0.13	20 ± 1.08
Amenorrhea	60.7 ± 0.12[a]	80.2 ± 0.17 [a]	52.21 ± 2.08 [a]

Values were expressed as means± SE. "a" superscript letter indicates a significant difference at ($P < 0.05$) when compared to normal group.

Changes in serum estrogen and progesterone hormones concentrations: Our results in table (2) showed a significant increase in serum estrogen and progesterone hormones concentrations in amenorrhea group as compared to normal females at ($P < 0.05$).

Table (2): Changes of estrogen and progesterone:

Group	Estrogen (pg/m L)	Progesterone (ng/ mL)
Normal group	200 ± 2.21	20 ± 1.21
Amenorrhea	18.21 ± 0.65 [a]	10 ± 1.07 [a]

Values were expressed as means± SE. "a" superscript letter indicates a significant difference at ($P < 0.05$) when compared to normal group.

Changes in serum oxidative/ antioxidant status: Our results in table (3) showed a significant decrease in serum GSH, TAC concentration in amenorrhea group as compared to normal females at ($P < 0.05$). Serum MDA showed a significant increase in amenorrhea group as compared to normal females at ($P < 0.05$).

Table (3): changes of oxidative/ antioxidant status:

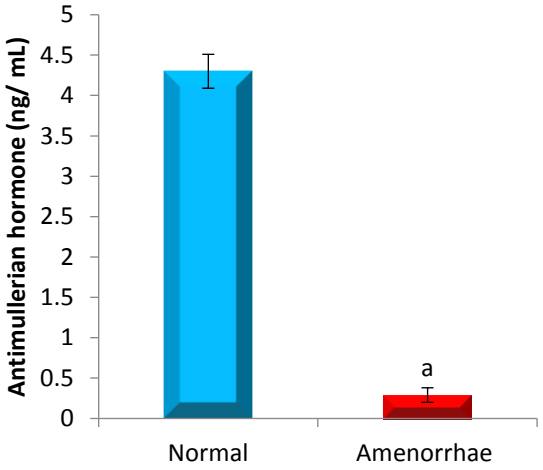
	GSH (µmol/ L)	TAC (mM/ L)	MDA (µmol/ L)
Normal	900 ± 2.98	2500 ± 2.09	1.21 ± 0.65
Amenorrhea	350 ± 1.87 [a]	970 ± 1.77 [a]	9.65 ± 1.02 [a]

Values were expressed as means± SE. "a" superscript letter indicates a significant difference at ($P < 0.05$) when compared to normal group.

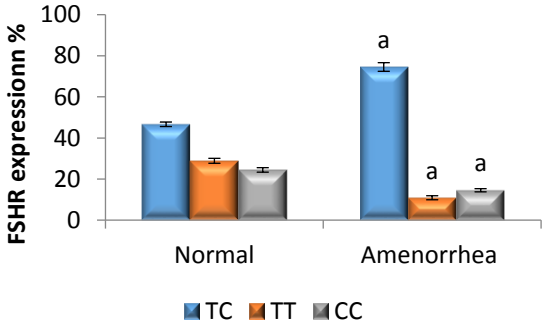
Changes in serum Antimullerian hormone (AMH) concentration:

Our results in figure (1) showed a significant decrease in serum AMH concentration in amenorrhea group as compared to normal females at ($P < 0.05$).

Figure (1): changes of Antimullerian hormone (AMH):



Changes in FSHR: our results showed a significant increase of heterozygous (TC) in amenorrhea as compared to the correspondence in normal at $P < 0.05$. Moreover, Homozygous (TT and CC) expressions of amenorrhea showed a significant decrease as compared to corresponding normal at $P < 0.05$.



a superscript letter indicates a significant difference at $P < 0.05$ as compared to the corresponding column of normal.

Discussion:

Primary amenorrhea remains a challenging problem for the gynaecologists as well as the patients and their families. It can affect the marital, sexual, reproductive and social status of the patient. Sometimes sex of raring becomes a problem. It needs a multidisciplinary team including gynaecologist, endocrinologist, plastic surgeon, psychiatrist and genetic counsellor for the management of patient. A proper protocol for investigation is important to avoid unnecessary investigations. Management plan should be according to cause, marital status, sex of raring. Counselling remains the main component of management. As for as the cause of primary amenorrhea [24]

A variety of disorders in the hypothalamus-pituitary-ovarian axis can lead to primary amenorrhea with delayed, arrested or normal pubertal development. Etiologies can be categorized as hypothalamic or pituitary disorders causing hypogonadotropic hypogonadism, gonadal disorders causing hypergonadotropic hypogonadism, disorders of other endocrine glands, and congenital utero-vaginal anomalies. This article gives a comprehensive review of the etiologies, diagnostics and management of primary amenorrhea from the perspective of pediatric endocrinologists and gynecologists. The goals of treatment vary depending on both the etiology and the patient; with timely etiological diagnostics fertility may be attained even in those situations where no curable treatment exists [25].

Gonadotrophins (FSH and LH), gondotrophins' receptors such as FSHR gene have key convergent and complementary roles in the ovary as major regulators of follicle development and ovarian function [26]. In the present study, to unravel the interaction between FSH, LH, FSHR, AMH and FSHR gene in female fertility, we examined two groups of women, normal and amenorrhea .

FSH affects follicular growth, maturation, dominant follicle selection as well as estradiol production [14]. It is considered to be an important survival factor for follicles in the course of folliculogenesis. FSH dampens apoptosis of cultured granulosa cells in vitro and protects follicles from atresia in vivo [27]. The scope of its function is very wide: it can inhibit atresia in follicles of different maturity including antral follicles, preovulatory follicles and dominant follicles and also in too many species to be listed here [28]. Our results showed that FSH and LH significantly increased in amenorrhea females compared to normal female. This findings agreed with Rebar et al., who reported a significant increase of both FSH and LH concentrations in primary amenorrhea compared to normal female and

this could be attributed to chromosomal abnormalities in females of primary amenorrhea that secondary amenorrhea [29]. The estrogen and progesterone levels were significantly reduced in amenorrhea females compared to normal female and this finding comes in agreement with Allaway et al., [30]. The reduction of estrogen and progesterone could be attributed to the gonadal dysgenesis which represents the most well-known cause of primary amenorrhea (30 % - 40 %) [31].

Antioxidant defenses protect the body from free radical-induced damage. The antioxidant defense system includes enzymatic (catalase, superoxide dismutases, glutathione peroxidase and glutathione reductase) and non-enzymatic [vitamin E, vitamin C, glutathione (GSH) and uric acid] [32]. In this study, a significant reduction of GSH and TAC was reported with a significant increase of MDA in amenorrhea compared to normal female. This imbalance in the oxidative/antioxidant redox exposes women to many dangers and diseases especially coronary heart diseases [33, 34]. The reduction of antioxidant defenses such as GSH and TAC is associated with low estrogen in primary amenorrhea. Previous study reported a significant positive correlation between estrogen level and antioxidant status in women during different stages of menstrual cycle [35]. Chang et al., stated that estrogen upregulates the expression of many antioxidants such as GSH [36]. Therefore, the low estrogen level in amenorrhea could be the logic cause of the recorded reduction of the antioxidant status with the concurrent increase of the oxidative stress which represent by MDA.

AMH plays a critical role in regulating ovarian function during reproductive life, in particular by limiting FSH actions in small growing follicles to prevent their premature maturation [17]. This study is, to our knowledge, the first to investigate the interplay between AMH and FSH in amenorrhea. Moreover AMH is a good indicators for many ovarian disorders [37]. This study could provide us with the opportunity to understand and decipher the hormonal dysregulation in amenorrhea. Our results showed a significant decrease of AMH concentration in amenorrhea compared to control group. This reduction could be a subsequent effect of the high level of FSH [38, 39].

FSHR mutations induce amenorrhea via ovarian failure [40]. The FSH resistance impaired follicular maturation [13]. Consequently, a hypersecretion of gonadotrophic hormones and a hypo-secretion of estrogen are developed as a result of FSHR mutations. Moreover FSHR mutations are associated with hypo-secretion of AMH. In patients with a FSH receptor mutation [41].

The genetic polymorphisms of the gonadotropins and their receptors especially FSHR play a pivotal role in the ovarian activity and regulation of menstrual cycle [42]. In the current study, we investigated FSHR polymorphism in Egyptian women with a primary amenorrhea. Our obtained data showed a significant increase of the heterozygous polymorphism (TC) of FSHR in women with amenorrhea compared to normal women. Moreover, the expressions of homozygous FSHR (TT and CC) were significantly lower in amenorrhea compared to normal women. These findings come in accordance with Achrekar and Modi who reported that FSHR gene polymorphism was detected in Finnish women with amenorrhea due to ovarian dysgenesis [43]. On the contrary, many other reports recorded no detected mutation in FSHR in American, German, Brazilian, Mexican and Argentine women with amenorrhea [44]. The previously mentioned studies strengthened the observation that CT mutation is restricted to Finnish population but our results contradict with this observation as we recorded CT mutation in Egyptian women with primary amenorrhea. Another previuos report has failed to detect any difference in the prevalence of FSHR genotype in both normal and amenorrhea women [45]. Any FSHR mutation altered the ability of the FSHR to bind FSH and to induce signal transduction pathway. Thus, loss of ovarian function in women is the direct result of the of impairment of the receptor function [46].

Conclusion

Our findings suggest that screening Egyptian women with amenorrhea from Upper Egypt indicated significant increase of LH, prolactin concentration with a marked hypo-secretion estrogen, progesterone and AMH. Moreover, a hypersecretion of FSH and FSHR polymorphism TC were provoked findings in women with primary amenorrhea. Women with amenorrhea showed an obvious imbalance of antioxidant/ oxidant redox. Further investigations are required to determine any other mutations in other receptors in order to fully understand the genotypic alterations in amenorrhea.

Supplementary Materials: supplementary materials will be available when requested.

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