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# European Gynecology & Obstetrics

# European Gynecology & Obstetrics

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# **EDITORIAL**

David Serfaty

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### WHY MALE MODERN CONTRACEPTION?

The Food and Drug administration (FDA) approved the contraceptive pill for women on June 23, 1960, a milestone that may be considered the second most important landmark in the life of women in the 20th century (following the recognition of voting rights). Today, almost 60 years on, men who wish to control their fertility must rely on female compliance with contraceptives or use a condom, withdrawal, vasectomy or periodic abstinence. Is it conceivable, in 2019, that the condom should still be the only method of male reversible contraception that exists? Regardless of the advances made with regard to condom use (such as the diversity of types available and the recent introduction of reimbursement of prescription-bought condoms by the French National Health Service), this method interferes with each act of intercourse and may decrease sexual pleasure. In addition, it must be correctly used to be fully effective.

Yet, numerous surveys indicate a strong interest in male contraception and numerous studies have shown efficacy of male hormonal contraceptives under development.

However, over the past 15 years, the pharmaceutical industry has jettisoned most of its investment virtually ceased investing in the field of male contraception, leaving only non-profit organizations and public entities pursuing male contraception research.<sup>[1]</sup>

#### Why should we fight for male contraception?

Involving women's partners in family planning services may be one avenue to reduce rates of unplanned pregnancy. But reducing unwanted pregnancies (and voluntary abortions) is not the only objective behind efforts to involve men in contraceptive use. Another important goal is to promote gender equality and enable men to take responsibility for their sexual and reproductive behavior.

# Research in male contraception: what is on the horizon? [2-6]

A drug called RISUG<sup>®</sup> (reversible inhibition of sperm under guidance) marketed as Vasalgel<sup>®</sup>, which the Parsemus Foundation likens to a reversible vasectomy, is certainly on the near horizon. This method entails injecting, into the vasa deferentia, a device composed of a high molecular weight polymer. This device remains in a soft gel-like state that allows water-soluble molecules to pass but not sperm. When men decide they no longer want to avoid pregnancy they receive a second injection (sodium bicarbonate) to dissolve the polymer. This method provided effective reversible contraception in rabbits and apparently in rhesus monkeys. The first clinical trial of Vasalgel<sup>®</sup> was announced in 2018.

#### Hormonal male contraception

A high intratesticular testosterone (T) concentration is required to support spermatogenesis. Administration of exogenous steroids (androgens alone or in combination with a progestin) suppresses testicular T production. Below a threshold amount of testicular T, sperm production does not take place.<sup>[2]</sup>

The following two hormonal male contraceptive methods under development appear to be the most promising:

- 1. The combined nestorone-testosterone gel for men. A phase IIB study (Efficacy and Safety Multicenter Study), approved by FDA, was launched in November 2018 by the National Institute of Child Health and Human Development (NICHD) and the Population Council https://www.popcouncil.org/ news/first-trial-launches-to-test-effectiveness-of-male-contraceptive-gel
- 2. A male contraceptive pill using a progestogenic androgen, dimethandrolone 17 $\beta$ -undecanoate (DMAU), may be available in the next decade, or one using 11 $\beta$ -methyl- nortestosterone-dodecylcarbonate (11 $\beta$ -MNTDC) also seems very promising <sup>[2]</sup>.

#### Non-hormonal approaches to male contraception

Several non-hormonal approaches to male contraception are also under development <sup>[2,7]</sup>: inhibitors of retinoic acid (essential for initiation of meiosis in spermatogenesis), BRDT (the bromodomain protein family is critical for chromatin remodeling during spermatogenesis), EPPIN (epididymal protease inhibitor added to the sperm surface), and gamendazole or Adjudin<sup>®</sup> (indazole carboxylic acid derivatives) may enter the clinical testing stage within a few years and become available about 10 years later.

In September 2013 an International Consortium dedicated to Male Contraception (ICMC) (www.ic-mc.info) was established in Paris. The ICMC is a network dedicated to all medical and socio-cultural aspects of male contraception, current and future, hormonal and non-hormonal, medical and surgical.

It currently has 114 members coming from 44 countries. Most of the leading researchers in male contraception worldwide are ICMC members.



The Consortium operates under the auspices of the European Society of Contraception, the European Society of Endocrinology, the Population Council, the Male Contraception Initiative, the Société Francophone de Contraception, the Association Française pour la Contraception, the European Society of Gynecology, and most recently, the European Academy of Andrology. The ICMC has organized two international congresses on male contraception (4 March 2016 and 7 May 2018), both held at the prestigious National Academy of Medicine in Paris. A «Paris Manifesto» was established after each congress. These manifestos were translated into several languages, including English, German French, Spanish, Portuguese, Chinese <sup>[7]</sup>, Hebrew and Arabic, and have been published in several medical journals. The programs of these past congresses as well as these «manifestos» are available on the ICMC website (www.ic-mc.info).

The third International Congress on Male Contraception will take place on May 11,2020, again at the National Academy of Medicine in Paris.

In addition, the ICMC has staged several scientific sessions on male contraception during European and other international congresses. Our aim is to continuously raise awareness about the field of male contraception and its progress.

Another important goal, as an advocacy group, is to prepare public, in particular medical, opinion for the much awaited advent of effective, reliable and well tolerated modern male contraceptives that may coexist harmoniously with the well-established modern forms of female contraception used worldwide.

I hope that the ICMC initiative, which highlights the state of the art in male contraception research, convening the international experts in this field at regular intervals, and addressing an area of family planning sidelined until now, will be successful in achieving its goals.

In any case, the time has come to address men's willingness to limit their family size and control their own fertility.

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## **Premature Ovarian Insufficiency**

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#### ABSTRACT

Premature ovarian insufficiency (POI) is a clinical syndrome defined by the presence of ovarian dysfunction before the age of 40 years. The prevalence of POI is around 1%. The following diagnostic criteria are mostly adopted nowadays: (i) oligo/amenorrhea for at least four months, and (ii) FSH levels > 25 IU/I on two occasions more than four weeks apart. POI can be the result of iatrogenic, genetic and autoimmune causes. Karyotyping should be performed in all women with non-iatrogenic POI, while fragile X premutation testing is also indicated. Screening with anti-21OH Abs (or alternatively adrenocortical Abs) and anti-TPO Abs should also be considered. No causal relationship between smoking and POI has been proved, but smoking has been associated with early menopause. In the majority of cases, the cause of POI is not identified and these women are described as having idiopathic POI. Untreated POI is associated with increased risk of type 2 diabetes mellitus and reduced life expectancy, largely due to cardiovascular disease. POI is also associated with reduced bone mineral density and increased risk of fracture. These women should maintain a healthy lifestyle with appropriate diet and exercise. Women with POI should also receive hormone replacement therapy with standard doses of oral (17B-E, 2-4 mg or CEE 0.625-1.25 mg) or transdermal (17B-E, 50-100 µg) estrogens and progestogens (natural progesterone 200 mg or dihydrogesterone 10-20 mg or norethisterone 1-5 mg) up to the age of normal menopause (50 years). There is a small chance of spontaneous pregnancy, therefore women with POI should be advised to use contraception, if they wish to avoid pregnancy. There are no interventions that have been reliably shown to increase ovarian activity and natural conception rates, therefore oocyte donation is, so far, the established option in the event of fertility issues.

#### **KEYWORDS**

Premature, ovarian, insufficiency, FSH, amenorrhea, oligomenorrhea, HRT.

#### Introduction

Premature ovarian insufficiency is a clinical syndrome defined by the presence of ovarian dysfunction before the age of 40 years <sup>[11]</sup>. It was first described in the early 1940s by Dr. Albright, a Harvard endocrinologist, who, at the time, called it primary ovarian insufficiency <sup>[2]</sup>. The word "primary" refers to the level of the defect, in this case the ovary, while "premature" refers to the timing, in this case before 40 years of age. Furthermore, the term "failure" has also been extensively used. However, as some of these women may present recurrent ovarian function, "insufficiency" is more appropriate. Therefore, the term premature ovarian insufficiency (POI) should be used to describe this condition, both in the clinical and in the research setting <sup>[1,3,4]</sup>.

The aim of this review is to present the current diagnostic criteria of POI and the prevalence of the syndrome, as well as to list the possible causes of this clinical condition. Furthermore, the authors discuss the appropriate diagnostic assessment approach, consider the short-term and long-term complications, and conclude with the appropriate combined therapeutic strategies for these women.

#### Article history

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#### **Diagnostic criteria**

POI is characterized by menstrual disturbances before the age of 40 years accompanied by raised gonadotrophins and low estradiol concentrations. The latter lead to estrogen deficiency symptoms, such as hot flashes, night sweats and sexual dysfunction. Hormonal confirmation is needed. Although proper diagnostic accuracy is lacking, the following diagnostic criteria are mostly adopted nowadays: (i) oligo/ amenorrhea, which means menstrual cycles longer than 35 days or absence of menses for at least four months, and (ii) FSH levels > 25 IU/l measured on two occasions more than four weeks apart. Anti-Mullerian hormone is an indicative marker of ovarian reserve, but it should not be routinely used for the diagnosis of POI <sup>[1,4]</sup>.

#### Prevalence

It is estimated that approximately 1% of women present POI worldwide, while the prevalence is estimated to be around 0.1% in women under 30 years of age, and around 0.01% in the under 20s. This varies according to specific population characteristics, such as ethnicity. For example, Afro-American women present POI in higher percentages (around 1.4%), while the prevalence is much lower in women of Japanese origin (around 0.1%) <sup>[5,6,7]</sup>. The cut-off age of 40 years is two standard deviations below the age of normal natural menopause in the western world, which is around 50 years <sup>[1,3]</sup>.

If a woman experiences menopause after 40 and before 45 years of age, this situation is called early menopause. In clinical practice, women in these two groups (menopause before 40 or between 40 and 45 years) often receive similar advice in terms of hormone replacement therapy (HRT), and cardiovascular and bone disease risk <sup>[1,3,4]</sup>.

#### Etiology

POI could be the result of iatrogenic, genetic or autoimmune causes. However, in the majority of cases (50-90%) the cause is not identified and these women are described as having unexplained or idiopathic POI. An association of smoking with early menopause has been described, however no causal relationship between smoking and POI has been proved <sup>[1]</sup>.

POI presents often after medical interventions affecting the ovaries, such surgery, chemotherapy and radiotherapy <sup>[5, 8, 9]</sup>. Some rather promising developments in the oncology world have, however, resulted in increases in the numbers of women with iatrogenic POI in recent years. First of all, there has been a significant improvement in the prognosis of childhood cancers over the last two decades, with long-term survival rates of more than 80% <sup>[5, 8]</sup>. In addition, an increasing number of premenopausal women carrying the BRCA gene mutation nowadays undergo risk-reducing surgery including prophylactic oophorectomy <sup>[5]</sup>.

Chemotherapeutic agents can have a direct toxic effect on the ovaries. This varies with different agents and is more common with alkylating agents, which can result in POI in approximately 40% of treated cases. The risk is also influenced by the dose of medications and the age of the woman at the time of treatment <sup>[10, 11, 12]</sup>. The toxic effects of radiotherapy are mostly related to the site of the treatment and are more common with pelvic, abdominal and whole body irradiation. The effect of radiotherapy is also dose and age dependent <sup>[8, 13]</sup>.

Genetic causes of POI include chromosomal abnormalities, mainly of the X chromosome, such as Turner syndrome or mosaic for Turner syndrome. Some cases with gonadal dysgenesis and the presence of Y chromosomal material can be also detected. Of course, such chromosomal abnormalities are mainly already present early in life, causing often primary amenorrhea <sup>[14, 15, 16]</sup>. Fragile X premutation is also present in quite a large percentage of cases, especially with familial POI. More specifically, the prevalence of POI in female carriers of fragile X premutation is between 13% and 26%. Moreover, 0.8% to 7.5% of women with sporadic POI are carriers, while in women with familial POI this percentage can be as high as 13% <sup>[14, 16, 17]</sup>. The fragile X mental retardation 1 (FMR1) gene is located on the long arm of the X chromosome. Full mutation of this gene causes fragile X syndrome, which presents with a broad spectrum of intellectual disability, hyperarousal, social difficulty, anxiety, aggression and autism.

POI is associated with a premutation, implicating expansion of a cytosine-guanine-guanine (CGG) trinucleotide repeat sequence in the first exon and promoter of FMR1. Normal alleles have 5 to 44 CGG repeats, intermediate alleles 45 to 54 repeats, premutation presents with 55 to 200 repeats, while the presence of more than 200 CGG repeats is consistent with full mutation <sup>[17]</sup>. Some other rare autosomal genetic causes associated with POI have been described, but these are usually part of generalized syndromes, such as the blepharophimosis ptosis epicanthus inversus syndrome <sup>[16]</sup>. Moreover, recent data deriving from animal genetic models, next generation sequencing studies and genome-wide association studies have indicated possible mutations and polymorphisms associated with POI in more than 60 candidate genes, again with high genetic heterogeneity <sup>[18, 19]</sup>.

POI could also be the result of autoimmune destruction of ovarian tissue <sup>[1,4]</sup>. In this case it is associated mainly with adrenal autoimmunity, and secondarily with thyroid autoimmunity. Specifically, 60-80% of cases with autoimmune POI may present positive adrenal autoantibodies, while 20-30% positive thyroid autoantibodies <sup>[20]</sup>. The latter condition is itself, of course, very common nowadays in the form of Hashimoto's autoimmune thyroiditis. Type 1 diabetes can also be associated with POI, but in smaller percentages of cases (2-3%). In the event of coexistence of two or more autoimmune endocrine disorders in the same patient, there must be a high suspicion of autoimmune polyendocrine syndrome <sup>[20, 21]</sup>.

Environmental chemicals may also disrupt female reproductive function. Adverse effects of endocrine disruptors on animal ovaries have been described, while serum levels of bisphenol-A and phthalate metabolites have been found to be increased in women with POI. The possible effects of endocrine disruptors during pregnancy with female embryos shed light on the fascinating hypothesis of a fetal origin of adult POI <sup>[22]</sup>.

#### **Diagnostic assessment**

Chromosomal analysis (for Turner syndrome or Y chromosomal material detection) should be performed in all women with non-iatrogenic POI. Therefore, karyotyping is a front-line test for all these women. Fragile X premutation testing is also indicated, while autosomal genetic testing is not at present indicated in women with POI, unless there is evidence suggesting a specific mutation <sup>[1]</sup>. The measurement of anti-ovarian autoantibodies has not yet been validated and is not recommended. However, screening with anti-21 hydroxylase autoantibodies (anti-210H Abs) or alternatively adrenocortical autoantibodies and anti-thyroid peroxidase autoantibodies (anti-TPO Abs) should be considered in women with POI of unknown cause or if an autoimmune disorder is suspected. Patients with positive anti-21OH Abs should be investigated for possible adrenal insufficiency, while patients with positive anti-TPO Abs should be investigated for possible hypothyroidism. With regard to adrenal insufficiency, morning cortisol measurement is the test of choice, followed by a dynamic synacthen test, if needed. TSH measurement is the first-line test for hypothyroidism, followed by measurement of peripheral thyroid hormones (T3 and T4), if needed <sup>[1,4]</sup>.

#### Short- and long-term health consequences

POI has both short- and long term health consequences in affected women (Table 1). The former are the result of reduced endogenous estrogen concentrations and include vasomotor symptoms, such as hot flashes and night sweats, sexual dysfunction, decreased energy, and impaired memory and concentration. Sexual dysfunction is due to both decreased libido and vaginal atrophy. In the long term, women with POI have fertility problems and this is usually the main concern of both patients and physicians. However, if they remain untreated these women may develop type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) <sup>[1,3,4]</sup>.

A recent meta-analysis published by our group indicated that women with POI indeed present a 50% increased risk of T2DM <sup>[23]</sup>. More specifically, this meta-analysis included 191,762 women in total, including 21,664 cases with T2DM, and it was found that women with early menopause and POI are at higher risk of T2DM compared with those aged between 45 and 55 years at menopause (OR 1.15,95% CI 1.04-1.26, p = 0.003; I2 61%, p = 0.002 and OR 1.50,95% CI 1.03-2.19, p = 0.033; I2 75.2%, p = 0.003, respectively). Similar differences emerged when women with early menopause and POI were compared with those aged >45 years at menopause (OR 1.12,95% CI 1.01-1.20, p = 0.02; I2 78%, p = 0.001 and OR 1.53,95% CI 1.03-2.27, p = 0.035; I2 78%, p = 0.001, respectively) <sup>[23]</sup>.

There is early evidence that these women are at higher CVD risk and this was confirmed by a recent, well performed meta-analysis, which included 10 studies, 190,588 women in total, with follow-up of 4 to 37 years and 9,440 events [2,026 ischemic heart disease (IHD), 6,438 stroke, 976 total CVD]. The researchers concluded that POI is associated with increased risk of IHD (HR 1.69, 95% CI 1.29-2.21, p=0.0001), and increased risk of total CVD (HR 1.61, 95% CI 1.22-2.12, p=0.0007), while no increased risk was found for stroke (HR

**Table 1** Short term and long term health consequences of women with

 Premature Ovarian Insufficiency (POI).

Short-term consequences	Long-term consequences
Vasomotor symptoms (hot flashes, night sweats)	Impaired fertility
Sexual dysfunction (low libido, vaginal atrophy)	Type 2 diabetes mellitus
Insomnia	Cardiovascular disease
Low energy	Osteoporosis and fractures
Impaired memory and concentration	Impaired cognitive function

#### 1.03, 95% CI 0.88-1.19, p=0.74) [24].

POI is also associated with reduced bone mineral density (BMD) and increased risk of fracture later in life. A recent meta-analysis published by our group included 462,393 women in total with 12,130 fractures; it emerged that the women with early menopause presented an increased fracture risk (OR 1.36, 95% CI 1.11–1.66, p = 0.002, I2 81.5%). No distinct effect on the site of fracture was found <sup>[25]</sup>. Last but not least, there is observational evidence of impaired cognitive function in the long term in women with POI <sup>[1,4]</sup>.

#### Management

The optimal management of women with POI should target both cardiovascular and bone health status. Therefore, these women should maintain a healthy lifestyle, which includes following a balanced diet with appropriate intake of calcium and vitamin D, doing aerobic and weight-bearing exercise, giving up smoking, reducing alcohol consumption, and maintaining a normal body weight <sup>[1, 26]</sup>. These lifestyle interventions are the cornerstone for prevention but also for treatment of T2DM and osteoporosis. Should they develop these diseases, most of them will eventually require pharmacological treatment, therefore appropriate agents should be chosen, taking into consideration their different metabolic, cardiovascular and bone health effects <sup>[26]</sup>.

Of course, POI should be treated as any endocrine deficiency problem and HRT is indicated. More specifically, standard doses of oral (17 $\beta$ -E2 2-4 mg or CEE 0.625-1.25 mg) or transdermal (17 $\beta$ -E2 50-100 µg) estrogens and progestogens (natural progesterone 200 mg or dihydrogesterone 10-20 mg or norethisterone 1-5 mg) are recommended up to the age of normal menopause [1, 3, 27], (Table 2). Women after hysterectomy should receive formulations with estrogens only, while in any woman with an intact uterus progestogen needs to be added. Oral progestogens are administered once or twice daily constantly, resulting in amenorrhea, or periodically 12-14 days per month, resulting in regular monthly bleeding. Transdermal progestogens (norethisterone) are administered twice weekly in a continuous manner, leading to amenorrhea, or in a cyclical manner, for 14 days a month, leading to regular monthly bleeding [3, 27].

HRT presents numerous beneficial effects for these women, including decreased lipid concentrations, improved body fat composition, increased insulin sensitivity and vascular function, as well as decreased CVD risk. Moreover, HRT amelio-

Table 2 HRT for women with POI (up to the age of 50 years).

Dosage		Oral estrogens			Transdermal 17B-E2		
Standard dose		176-E2 2- or CEE 0.6	4 mg 25-1.25 mg	50-100 µg			
Estrogens Dose	Natural progesterone		Dihydrogesterone		Norethisterone		
Standard dose	200 mg		10-20 mg		1-5 mg transdermal 0.25 mg		

POI: premature ovarian insufficiency; E2: estradiol; CEE: conjugated equine estrogens

rates BMD and decreases fracture risk in later life [<sup>28, 29, 30, 31, 32]</sup>. There have been some concerns with HRT regarding breast cancer and venous thromboembolism (VTE) risk, but these derive mainly from studies in older, naturally menopausal women. Indeed, observational data have shown that women with POI present a lower risk of breast cancer compared with controls, and HRT does not appear to increase the risk of breast cancer in women under the age of 50 years. As regards VTE, there is a lack of evidence in women with POI. Findings from studies in older menopausal women should not be directly extrapolated. However, the transdermal route of estradiol administration should be considered in women with POI who are at increased risk of VTE, such as obese ones [<sup>33, 34, 35]</sup>.

Regarding fertility, women with POI can have intermittent ovarian activity and there is a small chance of natural conception (~5%). Therefore, women with POI should be advised to use contraception, if they wish to avoid pregnancy <sup>[36]</sup>. There are no interventions that have been reliably shown to increase ovarian activity and natural conception rates. Therefore, oocyte donation is, so far, the established option for fertility issues in these women <sup>[1, 36, 37, 38]</sup>. However, oocyte or embryo cryopreservation should always be advised for women at increased risk of POI, such as before chemotherapy or radiotherapy <sup>[10, 11, 38, 39]</sup>. Last but not least, gonadectomy should be recommended for all women with detectable Y chromosomal material because of the increased risk of malignancy <sup>[1]</sup>.

#### Conclusions

POI is a rather common endocrine disorder and the prevalence of iatrogenic causes, in particular, looks set to rise. Women with POI suffer both short- and long-term health consequences, as a result of estrogen deficiency and oocyte depletion. They should follow a healthy lifestyle and be treated with standard doses of HRT up to the age of normal natural menopause. Oocyte donation is, so far, the only established option for fertility issues in women with POI, but oocyte or embryo cryopreservation should be recommended to all women before any intervention that may affect ovarian tissue.

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## Orgasm - a psychophysiology-based approach to hormonal and non-hormonal medical strategies to manage orgasmic disorders in women

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#### ABSTRACT

**Background:** Orgasm is a complex psychophysiological component of the human sexual response that involves different organ systems and central nervous system regulatory processes.

As part of the human sexual response, it is strongly linked to sexual desire and arousal.

With regard to hormonal and non-hormonal pharmacological approaches, most studies reporting treatment effects on orgasmic dysfunction are in women with concomitant desire and arousal disorders. On the basis of their action, these approaches can be divided into the following types.

• Enhancement of the afferent part of the spinal orgasmic reflex by improving the intensity of the sexual stimulus and/or increasing the receptivity to the stimulus (this demands structural integrity of vulva and vagina, blood flow to the vulva and the vagina)

These effects are provided by local estrogens, testosterone, Dihydroepiandrosterone (DHEA), non-hormonal lubricants, medicaldevices.

- Enhancement of the efferent pathway by noradrenergic/cholinergic activation and/or enzymatic action on the guanosine monophosphate (GMP) system.
- Drugs acting at this level are mainly PDE-5 inhibitors, vasodilators, specific prostaglandins
- Medical interventions (drugs) targeting brain centers which either increase central excitation or decrease nervous inhibition

Systemic estrogen/androgens act via central nervous receptors directly and indirectly through activation of neurotransmitters; centrally-acting drugs include bupropion, buspirone, flibanserin and melanocortin

These approaches can also be used in women without desire and arousal disorder.

In addition to these approaches, for women experiencing sufficient arousal but reporting inhibition at the plateau level, medical devices like Eros and Fiera have shown pro-orgasmic effects.

#### **KEYWORDS**

Female orgasm disorder, neurophysiology, medical treatment.

#### Introduction

Orgasm can be understood as a complex summation reflex that is regulated by both the somatic and autonomic nervous systems linked to central nervous processing regions.

On the basis of this concept two major approaches in research and clinical practice can be distinguished, which serve as the basis for diagnosis and therapy. One sees orgasm as a biological process involving different organ systems and networks, while the other sees it as a subjective experience.

# a) Orgasm as a biological process involving different organ systems and networks $^{\left[ 1-4\right] }$

#### Afferent pathways

The pudendal nerve relays sensory stimuli from the external genitals, the perineum, clitoris and urethra, and the pelvic floor musculature. The pelvic and hypogastric nerves mediate sensory information from the internal pelvic organs. Light touch, noxious and/or chemical stimuli of the vulva, vagina, cervix,

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and uterus are primarily mediated via the pelvic nerve. Another afferent pathway in animal research is the vagus nerve which conveys sensory information from female pelvic organs to nuclei in the brainstem.

It is important to note that these main afferents are sensitive to the levels of steroid hormones.

#### Spinal centers

The pudendal nerve afferents enter the spinal cord through the superficial dorsal horn of segments L6-S1 (in humans S2-S4) and travel through the medial dorsal horn to the dorsal gray



commissure which is located in the medial cord. The hypogastric nerve afferents terminate in the medial dorsal horn and medial gray of spinal segments T13-L3. The spinothalamic and spinoreticular pathways relay sensory information to the brain. Descending pathways travel through the same structures.

#### Efferents mediating genital responses

The efferent fibers of the pudendal nerve provide innervation of the pelvic floor and anal and urethral sphincters. The pudendal motor neurons are located in the ventral horn of the lumbar spinal cord in Onuf's nucleus. The pelvic nerve is composed of parasympathetic preganglionic neurons (the sacral parasympathetic nucleus), located primarily in the lumbosacral spinal cord.

The hypogastric nerve is composed of sympathetic preganglionic neurons in the upper lumbar spinal cord. The preganglionic neurons arise from the medial and intermediolateral cell columns.

#### Supraspinal control

Inhibition

The nucleus paragigantocellularis (NPGC) exerts inhibition of spinal sexual reflexes in males and females. The NPGC projects directly to pelvic efferent neurons. Serotonin is the main neurotransmitter in this system with an inhibitory action.

Stimulation

The medial preoptic area (MPOA) plays an important role especially in males but also in females. Stimulation of this nucleus and injection of galanin into the MPOA facilitates some female sexual behaviors in animal research. There is no direct connection between the MPOA and the lumbosacral centers, but the MPOA sends information through a relay to hypothalamic and brainstem nuclei.

#### The endocrine environment

Sex steroids, interacting with receptors in the brain, provide the endocrine milieu. Estrogens (mainly estradiol) have central nervous system effects which seem to increase receptivity to sexual stimuli and facilitate permissive behavior in animal research. Peripheral effects of estrogens are important in maintaining vulvovaginal structural integrity and facilitating blood flow to the vagina. Testosterone has a central excitatory and activating effect on the limbic system, including brain centers linked to sexual motivation. In animal research this is related to proactive search for sexual clues. The role of progesterone is still not completely elucidated, but it seems to be directed mainly towards reproductive function. Circulating levels of prolactin, vasopressin, oxytocin, adrenalin and vasointestinal polypeptide have been reported to increase with orgasm. Prolactin increases with orgasm are maintained for approximately 60 minutes after orgasm.

#### Central modulatory input

Several nuclei in the brainstem including the NPGC, the raphe nuclei pallidus and the locus ceruleus project to pelvic efferent neurons and interneurons in the lumbosacral spinal cord, most likely to modulate lumbosacral spinal cord reflexes.

The periaquaductal gray matter of the midbrain is heavily interconnected with the brainstem and hypothalamic sites related to sexual behavior, seemingly serving as a relay center. Within the hypothalamus, the medial preoptic area, nucleus paraventricularis and the ventromedial nucleus are believed to have major roles in female sexual function. During orgasm, activation of the mesodiencephalic transition zone can be observed. Serotonin, dopamine, epinephrine, opioids are neurotransmitters and neuropeptides that modulate female sexual function. In the context of dual control of human sexual behavior, these molecules act either as excitatory signals (epinephrine, dopamine) or as inhibitory signals (serotonin, opoids)

#### b) Orgasm as a subjective experience

According to Mah and Binik, <sup>[5]</sup> the subjective experience of orgasm can be broken down into three elements: sensory, evaluative and affective.

- Sensory components are the build-up of tension, release of tension, spreading sensations, whole body involvement, ejaculatory sensations, rhythmic sensations, thermal sensations, miscellaneous.
- Evaluative components are the feeling of inevitability, temporal evaluation, intensity, physical effects, depth, global pleasure, sensual pleasure, satisfaction and excitement.
- Affective items are emotional intimacy, joy-peacefulness, joy-elation, emotional excitation, emotional fusion, unreality, lack of awareness of surroundings, suspension, miscellaneous.

#### **Orgasmic disorders**

Female orgasmic disorders (FOD) are absence of orgasm, difficulty experiencing orgasm, or decreased intensity of orgasm during all or most episodes of sexual activity. <sup>[6]</sup> The symptoms can be lifelong or acquired. Difficulty reaching orgasm might be isolated to specific sexual activities, situations or partners. The symptoms must be distressing to the individual.

A few subtle but important differences distinguish the diagnostic criteria for FOD as reported in the DSM-IV-TR and DSM-5. A significant change in the DSM-5 was the removal of the criterion requiring that difficulty with orgasm occur despite "a normal excitement phase." <sup>[6]</sup>

The diagnosis is thus based mainly on the subjective experience. The objective criteria are summarized by Meston *et al.*<sup>[7]</sup>

#### Medical treatment of orgasmic disorders

With regard to the medical treatment of orgasmic disorders, clinical situations can be divided into two types:

- medical treatment of orgasmic disorder in the context of desire/arousal disorders
- medical treatment of orgasmic disorder in women without desire/arousal disorders

In clinical practice the hormonal and non-hormonal pharmacological treatment of orgasmic disorders is based on studies that fail to differentiate clearly between these groups. Therefore treatment strategies and options overlap.

Medical treatment of orgasmic disorders in the context of desire/arousal disorders.

Most of the drugs summarized below are studied in the context of desire and arousal disorders. In this setting, orgasmic function is measured as a secondary outcome looking for an increase in orgasmic capacity (frequency, intensity). The following approaches can be distinguished:

Enhancement of the afferent part of the spinal orgasmic reflex by improving the intensity of the sexual stimulus and/or increasing the receptivity to the stimulus (structural integrity of vulva and vagina, blood flow to the vulva and the vagina).

Local estrogen therapy

Local estrogen therapy (LET) increases blood flow in the microcirculation of the vagina, leading to vasocongestion (experimental studies) [8] LET improves vulvovaginal integrity in menopausal women with vulvovaginal atrophy syndrome (VVA) and VVA-related sexual arousal and pain symptoms (RCTs, guidelines) [9-11]. The extent to which pain symptoms contribute to orgasmic dysfunction may not be easy to discern. · Local estrogen and testosterone

A clinical trial <sup>[12]</sup> showed improvement in all domains of the Female Sexual Function Index (FSFI) in women receiving local estrogen or testosterone compared to placebo lubricant.

• Dihydroepiandrosterone DHEA

Randomized controlled trials (by one group) [13,14] showed improvement of the integrity of the mucosa and the connective tissue and showed a positive effect on arousal. In a review Davis et al. [15] found no clear evidence suggesting a prosexual effect in postmenopausal women.

Enhancement of the efferent pathway by noradrenergic/cholinergic activation and or enzymatic action on the guanosine monophosphate (GMP) system.

Phentolamine

Phentolamine is an a1 and a2 adrenergic agonist tested in a small pilot study with a low level of evidence. [16] The participants were postmenopausal women with a lack of lubrication and with sexual arousal difficulties of at least 6 months' duration. All subjects received a single dose of oral phentolamine (40 mg) and placebo in a single-blind, dose-escalation design. Dependent variables for the study included vaginal pulse amplitude, measured by means of vaginal photoplethysmography, self-report measures of sexual response, and patient- and physician-based assessments of adverse events. The results indicated a mild, positive effect of phentolamine across all measures of arousal, with significant changes (p < .05) in self-reported lubrication and pleasurable sensations in the vagina.

Cholinergic drugs

Betanechol is a cholinergic agonist. It was investigated in male patients with depression treated with an SSRI (clomipramine) and suffering from ejaculatory delay.<sup>[17]</sup> 12 fully remitted panic disorder patients, complaining of severe clomipramine-induced ejaculatory delay, were randomly assigned to either bethanecol chloride tablets (20 mg, as needed) or placebo according to a randomized, double-blind, placebo-controlled, two-period crossover design. A visual analog scale was used to assess severity of the orgasmic dysfunction. A clear improvement was observed in the active treatment period. No placebo or carry-over effects were observed. To date, there are no published studies in females.

#### • PDE-5 inhibitors

These drugs increase vaginal blood flow via the GMP system (see above). One RCT, a randomized cross over study with sildenafil, showed improvement in arousal and orgasmic function.<sup>[18]</sup> The authors of a larger study reviewed a total of 16 studies. Studies using self-reported measures of sexual functioning showed mixed results whereas ones examining physiological effects of Phosphodiesterase-5 inhibitors PDE-5 on genital vasocongestion consistently reported significant effects on genital sexual response. [19]

• Topical alprostadil

This drug acts on vasodilation and vasocongestion in the vagina. A review found that in-clinic application of alprostadil increased genital vasocongestion, vaginal erythema, transudates, and some patient-assessed indices of sexual arousal; however, these effects were not consistently superior to placebo. Three out of 4 trials investigating at-home use of topical alprostadil demonstrated improvements in achievement of satisfactory levels of sexual arousal and successful sexual encounters in patients with female sexual arousal disorder (FSAD).<sup>[20]</sup>

Medical interventions (drugs) targeting brain centers that either increase central excitation or decrease central inhibition

- Systemic estrogen and testosterone therapy
- Systemic estrogen, systemic testosterone, and combined estrogen and testosterone therapy decrease arousal dysfunction, increase desire and improve orgasmic function in premenopausal and postmenopausal women and in women after unilateral or bilateral oophorectomy. These effects are due, on the one hand, to the permissive and receptive central nervous system effects and the positive effects on vulvovaginal structure and function of estrogens, and on the other to the activating and excitatory effect of testosterone on the limbic system of the brain (RCTs, level of evidence 1). [21-34]
- Tibolone

Combined estrogenic, progestogenic and androgenic action (RCTs, level of evidence 1), [35-38] increasing desire, arousal and orgasm.

Bupropion

Buproprion acts via dopamine and norepinephrine reuptake inhibition and does not have a direct serotoninergic effect (RCTs, mild to moderate effect). [39-41]

• Flibanserin

5-hydroxytryptamine (5-HT)1A agonist and 5-HT2A antagonist, binding also with moderate affinity to 5-HT2B, 5-HT2C and dopamine D4 receptors. RCTS and reviews show significant effect. [42-51]

• Buspirone

5-HT1 agonist possibly producing some oxytocin activation. Some evidence of prosexual effects in patients treated with SSRIs for depression. [52]

• Vilazodone

Partial HT1A agonist. (see above)

• Melanocortin agonists [53]

Bremelanotide is a melanocortin-3 and 4 agonist with an activating effect on arousal pathways (basic science studies, dose-finding studies, RCTs). Preliminary studies are ongoing; these show prosexual effects in men and women (Phase II studies)  $^{\left[54-58\right]}$ 

Apomorphine

Apomorphine is a non-selective dopaminergic receptor agonist (1 RCT showing prosexual effects) <sup>[59]</sup>. However, the occurrence of significant nausea and vomiting have prevented its further development.

#### **Drugs with unspecific effects**

Lady Prelox

20 mg Pycnogenol<sup>®</sup> pine bark extract, 200 mg L-arginine, 200 mg L-citrulline and 50 mg Rosvita<sup>®</sup> rose hip extrac (2 observational studies showing small group prosexual effects, low level of evidence).<sup>[60,61]</sup>

Lady Prelox is a dietary supplement which was given to 100 women in a healthy lifestyle intervention where sexual function was evaluated using the FSFI in part of the sample (30 women). This is an uncontrolled observation with very low level of evidence.

Gingko biloba

Gingko biloba may have an effect on blood flow (central, peripheral). An RCT failed to show a significant effect if when was applied alone but supportive effect in the context of counseling.<sup>[62]</sup>

ArginMax

This is a blend of Ginseng, Ginkgo biloba, damiana leaf and vitamins. (1 RCT showed it to be superior to placebo, but the difference was not statistically significant).<sup>[63]</sup>

#### Short-acting drugs for "on demand" treatment

Oxytocin

Oxytocin (OXT) may work synergistically with sex hormones to facilitate muscle contractions during orgasm. Oxytocin is secreted by the paraventricular nucleus of the hypothalamus into the bloodstream during arousal and orgasm, and it is thus considered a facilitator of arousal and orgasm. <sup>[64]</sup>

A study involving 29 healthy heterosexual couples (n=58 participants), studied in a naturalistic setting, explored the effects of intranasally administered OXT (24 IU) on sexual drive, arousal, orgasm and refractory aspects of sexual behavior together with partner interactions. Data were assessed using psychometric instruments (Acute Sexual Experiences Scale, Arizona Sexual Experience Scale) as well as biomarkers, such as cortisol,  $\alpha$ -amylase and heart rate.

Intranasal OXT administration did not alter "classical" parameters of sexual function, such as sexual drive, arousal or penile erection and lubrication. However, analysis of variance and a hierarchical linear model (HLM) revealed specific effects related to the orgasmic/post-orgasmic interval as well as parameters of partner interactions. According to HLM analysis, OXT increased the intensity of orgasm, contentment after sexual intercourse and the effect of study participation. According to ANOVA analysis, these effects were more pronounced in men. Men additionally indicated higher levels of sexual satiety after sexual intercourse with OXT administration. Women felt more relaxed and subgroups indicated better abilities to share sexual desires or to empathize with their partners. The effect sizes were small to moderate.<sup>[65]</sup> Thus, OXT can be considered a facilitator of arousal and orgasm. <sup>[64,65]</sup> The compound's instability at room and body temperature severely limits its utility under real-world conditions.

#### Combination of short-acting drugs on demand

This therapeutic concept is based on the different prosexual actions of testosterone (central effect), sildenafil (peripheral effect) and buspirone (dopaminergic and specific 5HT receptor action). Treatment combinations have been tested based on the dual control model, looking into differential effects of drug combinations in women with low and high inhibition. <sup>66,67]</sup> The combination of testosterone sublingually (0.5mg) with buspirone (10mg) showed a prosexual effect in women most probably by reducing inhibition. <sup>68]</sup> The combination of testosterone sublingually (0.5mg) with the PDE5-inhibitor sildenafil 50mg) also showed prosexual effects significantly over placebo, most probably mediated by activation of the peripheral response (excitatory effect). <sup>[69]</sup>

#### Medical treatment of orgasmic disorder in women with subjectively sufficient arousal

These treatments are based on studies performed in women with the main complaint of not being able to experience/attain an orgasm despite the fact that they feel desire and subjective excitement and arousal. There are very few studies that include women with an isolated orgasmic difficulty.

The underlying pathophysiology can be described as an interruption of or decline in the process of growing intensity of arousal towards the development of the orgasmic response beyond what has been described as plateau phase. Physiological treatments targets therefore consist of:

- Intensification of the arousal stimulus (strength, frequency, additional stimuli) through lubricants and moisturizers and specific sexual response-enhancing topical products. Zestra, for example, is an over-the-counter massage oil of a blend of borage seed oil, angelica extract, evening primrose oil, coleus extract and vitamins C and E, and it was designed to increase blood flow to the clitoris, labia and vaginal opening. Borage oil and evening primrose oil both contain high amounts of gamma-linolinic acid, which is metabolized to prostaglandin E1, angelica root contains osthole, which increases cGMP and cAMP, and the components of coleus extract are adenylate cyclase stimulants. In a small, randomized, double-blind placebo controlled, two-way crossover study [70] of 10 women with FSAD and 10 women without FSAD, this topical application was associated with increases in levels of arousal, desire, ability to have orgasm, and sexual pleasure and satisfaction, in both the normal and the FSAD-affected women when compared with placebo. Women with FSAD showed a greater response than women without the condition, and women using SSRIs had the same improvement as women not using antidepressants
- PDE5-inhibitors and prostaglandins. (as decribed above). The drug most used in studies is siledenafil and alprostadil (see above)

#### Medical devices

Medical devices may work through vibratory stimulation or by causing clitoral vascular engorgement using a vacuum system. While a number of vibratory stimulating devices are available, only one U.S. Food and Drug Administration (FDA) cleared-to-market device is available by prescription to treat FSD: the Eros<sup>®</sup> Therapy device (UroMetrics, Inc., St. Paul, Minn., USA). Eros<sup>®</sup> is a small, battery-powered appliance used to gently apply direct vacuum over the clitoris causing the clitoral erectile chambers and labia to fill with blood. The vibration from the suction pump may also aid in stimulation.

Fiera<sup>™</sup>

Fiera Arouser for Her<sup>™</sup>, abbreviated as simply-- Fiera<sup>™</sup> (Nuelle, Inc.) is the first wearable "intimacy enhancer". The device consists of a small, rechargeable, battery operated, vibrating device which "adheres" to a woman's clitoris by gentle suction (i.e. it is hands free). It is worn in preparation for sexual activity. The device is meant to decrease the time to orgasm by providing added stimulation time before foreplay.

#### Conclusions

Hormonal and non-hormonal pharmacologic strategies for managing orgasmic disorders in women are based on an understanding of the psychophysiology of the orgasmic phase of the human sexual response.

These strategies include enhancement of the afferent stimulatory signals of the orgasmic reflex through facilitation of the tissue and neurovascular response (local hormonal and non-hormonal treatments, medical devices), enhancement of the efferent action of neurovascular and neuromuscular activation (vasodilatation), modulation of the central processing response by increasing excitatory neurotransmission and decreasing inhibitory signals by means of centrally acting drugs (e.g. systemic estrogen and testosterone therapy and drugs interacting with serotonin, dopamine, noradrenaline, melanocortin, oxytocin and neurosteroid pathways).

There is good evidence that effective medical treatment of desire/arousal disorder increases the chances of orgasmic response in the individual woman.

With regard to the medical treatment of isolated orgasmic dysfunction more research and clinical trials are needed.

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# Mitochondrial DNA diseases: preimplantation diagnosis and intervention possibilities

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#### ABSTRACT

Mitochondrial DNA mutations are exclusively maternally inherited and can cause severe diseases for which there is no effective treatment. The recurrence risk of mitochondrial diseases is difficult to estimate due to heteroplasmy and the bottleneck effect during oogenesis. Here we review the literature on current options for preimplantation genetic diagnosis and interventions to prevent mitochondrial disease transmission.

Preimplantation genetic diagnosis can be performed in different developmental stages of the oocyte or the zygote. Preimplantation interventions consist of nuclear transfer, a set of techniques in which the patient's nuclear genetic material is placed in an enucleated donor cell, or genomic edition, through which the mitochondrial genome is altered. These methods are associated with technical barriers, such as ensuring the representativeness of the analysed sample when applying preimplantation genetic diagnosis, maintaining the communication between nuclear and mitochondrial genomes when using nuclear transfer, and avoiding off-target modifications when genome edition is the choice.

Although much has already been accomplished, further research is required to reduce the risk to the offspring and to develop more efficient and safer techniques.

#### **KEYWORDS**

DNA, Mitochondrial; Mitochondrial Diseases; Preimplantation Genetic Diagnosis; Reproductive Techniques, Assisted; Gene Editing.

#### Introduction

Mitochondria are the "powerhouses" of the cell because of their role in adenosine triphosphate (ATP) production.<sup>[1-4]</sup> Although mitochondrial deoxyribonucleic acid (mtDNA) accounts for less than 0.1% of the total cell DNA,[5] mtDNA mutations are responsible for most inborn metabolic diseases,<sup>[6-8]</sup> affecting preferentially the most energy demanding tissues.<sup>[2,9]</sup>

MtDNA mutations may be homoplasmic (only mutated mtDNA is present in all tissues) or heteroplasmic (characterised by variable proportions of normal and mutant mtDNA among cells and tissues).<sup>[10-12]</sup> Clinical manifestations occur only when the mutated mtDNA load exceeds a threshold that is both tissue and mutation specific,<sup>[1,7,13]</sup> although there is not always an exact genotype-phenotype correlation.<sup>[13-16]</sup>

The inheritance of mtDNA is exclusively maternal <sup>[1,17-19]</sup> and is affected by the genetic bottleneck <sup>[12,13,17,18,20]</sup> through which a few mtDNA molecules become founders of the off-spring. <sup>[21,22]</sup>

Considering the prevalence,<sup>[13]</sup> the high severity,<sup>[13,23]</sup> the absence of curative treatment,<sup>[11,13,18,24]</sup> and the high recurrence risk for the offspring of female carriers of these diseases,<sup>[13]</sup> prevention of their transmission would be of great importance. The aim of this review is to describe recent developments in this field.

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#### **Methods**

We conducted a review of Pubmed using the MeSH terms "DNA, Mitochondrial" and "Mitochondrial Diseases" combined with "Reproductive Techniques, Assisted" and "Preimplantation Diagnosis" and excluding the term "Infertility", and of Embase using the corresponding Emtree terms. Additional filters used were: Portuguese and English languages, humans and last ten years. "Conference Abstract", "Letter" and "Editorial" typologies were excluded. 133 journal articles were collected, and 77 were included for analysis and, when appropriate, included in the review.

# Methods for preventing the transmission of mtDNA diseases

#### 1. Preimplantation genetic diagnosis

The aim of preimplantation genetic diagnosis (PGD) is to transfer embryos after evaluating their mutation loads, which are variable due to the genetic bottleneck and random segregation during oogenesis.<sup>[15,25]</sup>

Mitochondria may be accessed for evaluation at different stages: the first polar body of the oocyte and the blastomeres of the cleavage stage or blastocyst stage embryos.<sup>[15]</sup> Analysing mtDNA is easier and less prone to artifacts than analysing nuclear DNA (nDNA) due to the higher number of mtDNA copies per blastomere.<sup>[7,14]</sup>

The first polar body biopsy is performed before fertilisation, which may be ethically acceptable to those opposed to embryo testing.<sup>[15]</sup> However, a low correlation between the mutation load of the polar body and the oocyte, probably due to the asymmetric segregation of mitochondria during meiosis, was described.<sup>[7,12,14,25]</sup>

One of the major challenges of the other available options is to ensure the representativeness of the mutation load of the sample.<sup>[10,23,25]</sup> There is no consensus on whether one or two blastomeres should be used when performing blastomere biopsy.<sup>[7]</sup> While one cell may be sufficient in most cases,<sup>[1,13,23]</sup> being less detrimental to the embryo's viability,<sup>[15,23]</sup> most authors suggest using two cells and considering the higher percentage of mutated mtDNA when discrepancies are found.<sup>[7,14]</sup>

In blastocyst biopsies, trophectoderm cells are collected, as they are judged to be representative of the inner cell mass of the embryo.<sup>[12,24,25]</sup> Several cells can be removed without a negative impact on embryo development,<sup>[7,25,26]</sup> allowing a more accurate prediction of the mutation load of the embryo.<sup>[7]</sup> Nevertheless, in this stage, the cell to cell variation is higher than that found with cleavage stage embryos.<sup>[23]</sup>

A mutation load below the threshold level is considered the criterion for choosing embryos to transfer.<sup>[7,13,17,19,21]</sup> Ideally only embryos with no mutated mtDNA should be used.<sup>[6,14]</sup> However, as the threshold is reduced, fewer embryos will be available.<sup>[6,11,15,21]</sup> Defining an appropriate threshold is still difficult because of the lack of available data.<sup>[6,13,15]</sup> Recent studies tried to set a threshold to be applied to all mtDNA mutations, 18% being the value obtained with 95% confidence.<sup>[6,7,14,19,24,27]</sup>

Obtaining embryos with an acceptable mutation load can require multiple ovulation stimulation cycles in order to find the best possible embryo.<sup>[7,14]</sup>

PGD cannot be used in women with homoplasmic mutations,<sup>[11,16,17,19,20,28]</sup> and is of limited value for women with a high mutation load <sup>[17,19,20]</sup> and for those whose mutations have a poor correlation between mutation load and disease severity.<sup>[1,17]</sup>

#### 2. Ooplasmic transfer (Figure 1)

When adequate embryos are not available, different approaches must be considered.

One option is ooplasmic transfer, which consists of injecting ooplasm with normal mitochondria from a healthy donor into an oocyte containing mutated mtDNA.<sup>[1,3,20,29–32]</sup>

It has been suggested that ooplasmic transfer would lead to a reduction of the effects of mtDNA mutations through a dilution effect,<sup>[31]</sup> but this is only a theoretical possibility.<sup>[17,30]</sup>

One of the barriers is that only up to 15% of donor ooplasm can be transferred, whereas a larger amount would be needed. <sup>[1,17,30,31]</sup> Other suggestions are to use purified mitochondria or to partially remove the mitochondria from the oocyte.<sup>[1]</sup> Another concern is the lack of information on the long-term effects of introducing a new mtDNA haplotype into the oocyte.<sup>[17,22]</sup> So far, multiple chromosomal abnormalities and birth defects have been reported, leading to this technique being banned.<sup>[1,3,17,32]</sup>

#### 3. Nuclear transfer

Nuclear transfer (NT) describes a set of techniques involving removal of mutation carrier nDNA, followed by its transfer to an enucleated oocyte from a donor, so as to obtain a new cell with nDNA from the patient and mtDNA from a donor. <sup>[4,7,20,22,28,31,33]</sup> It can be performed through five different techniques: germinal vesicle transfer (GVT), meiotic spindle trans-





fer (MST), pronuclear transfer (PNT), polar body transfer (PBT) and blastomere transfer.

MST and PNT have been allowed by the HFEA, which considers them potentially useful for patients whose offspring is at risk of severe mtDNA diseases and have no other option for having their own genetic children.<sup>[20,29,34,35]</sup>

Possible germline genetic modification associated with NT raises some concerns.<sup>[14,20,26,31,35,36]</sup> The Nuffield Council on Bioethics concluded that if these techniques are proven to be safe and effective, it would be ethical to use them due to the health and social benefits of a life free from mitochondrial disorders. <sup>[2,20,35]</sup> These techniques do not lead to a "third parent", either biologically or legally.<sup>[2,9,35,36]</sup>

A major concern is the risk of co-transference of mutated mtDNA.<sup>[7,8,11,20,21,28]</sup> Even if this occurs, low levels of mutated mtDNA would be expected, and usually not linked to disease manifestation.<sup>[7,20,35]</sup> However, there is a slight risk of mutated mtDNA segregation to specific tissues.<sup>[3,11,20]</sup>

Recent studies showed that the chance of disease recurrence in subsequent generations is dramatically reduced if a mutant load below 5% is achieved.<sup>[17,36]</sup> There are also concerns over the possibility of one mtDNA haplotype replicating faster than the other,<sup>[17,37–39]</sup> enabling transformed embryos to "revert" to a damaged condition.<sup>[34,40,41]</sup> This seems more likely to occur when large DNA sequence differences exist between haplotypes.<sup>[22,38]</sup>

The consequences of possible mismatch between the patient's nuclear genome and the donor's mitochondrial genome has been studied by comparing differentiation efficiency, mitochondrial enzymatic activity and oxygen consumption rate between cell lines grouped based on single nucleotide polymorphism differences between the patient and the donor's oocyte mtDNA. Similar results were obtained in the different groups, suggesting that compatibility exists.<sup>[4,40]</sup>

The main drawback for the clinical application of NT is its inefficiency.<sup>[1,21]</sup>

MST was applied successfully in 2016, resulting in the birth of a male child with reduced levels of pathogenic mtDNA.<sup>[33,42]</sup>



In the germinal vesicle stage, mitochondria are concentrated in the peri-nuclear space, which can lead to co-transfer of a significant amount of mutated mtDNA.<sup>[3]</sup>

Germinal vesicle removal is less invasive compared with other procedures.<sup>[30]</sup> A major disadvantage is the requirement of *in vitro* maturation of the oocytes,<sup>[1,12,17,30]</sup> which is still an inefficient procedure.<sup>[1,17,27]</sup>

#### **b. Meiotic spindle transfer** (Figure 3)

The visualisation of the spindle requires the use of polarised light microscopy.<sup>[12,17,20,32]</sup> Its removal is also difficult <sup>[2]</sup> and a certain volume of ooplasm has to be co-transferred to prevent chromosome loss.<sup>[43]</sup> However, as mitochondria are scattered in the ooplasm,<sup>[3]</sup> this technique is associated with minimal carry-over.<sup>[12,14,20,44]</sup>

Most studies showed that fertilisation rates after this technique were similar to those observed in controls.<sup>[35,40]</sup> Nevertheless, the spindle is very sensitive to micromanipulation,<sup>[43,44]</sup> which frequently induces premature activation of oocytes.<sup>[16,20]</sup> This can lead to abnormal fertilisation due to premature chromatid separation in the absence of the second polar body, resulting in a high incidence of abnormal numbers of pronuclei. <sup>[12,14,32]</sup> It is also crucial to remove the first polar body from the donor oocyte because it can be reabsorbed, causing polyploidy. <sup>[32]</sup> Despite all these possible complications, aneuploidy rates seem to be similar to those found in controls.<sup>[27,35,40]</sup>

#### c. Pronuclear transfer (Figure 4)

This technique requires fertilisation of the donor and the recipient oocytes.  $^{[20,28,30,35]}$ 

Pronuclei are easier to manage because of the larger volume and membrane, but its removal causes greater cellular trauma.<sup>[2]</sup>

In the pronuclear stage, mitochondria are concentrated in the peri-nuclear space, which may lead to higher mutated mtDNA carryover.<sup>[3,45]</sup> Available data on heteroplasmy is not consistent, some studies reporting over 20%,<sup>[2,37,43]</sup> and others



Figure 2 Schematic representation of germinal vesicle transfer.

Figure 3 Schematic representation of meiotic spindle transfer.



Figure 4 Schematic representation of pronuclear transfer.



less than 2%.<sup>[12,20,32,44]</sup> The high percentages observed in some studies are justified by mtDNA amplification around pronuclei induced by zygotic activation.<sup>[43]</sup>

Studies to date have shown low embryonic development, but further investigation is required in order to clarify whether this is a consequence of the technique or of using abnormally fertilised embryos for testing.<sup>[20]</sup>

The major disadvantage of this procedure is that half of the embryos created will be discarded.<sup>[20,30,43,44]</sup>

#### d. Polar body transfer

Polar body transfer (PBT) is similar to MST or PNT when performed using the first or the second polar body, respectively.<sup>[44]</sup>

Polar bodies theoretically share the same genetic information as the oocyte,<sup>[43,44]</sup> but contain very few cytoplasm and cellular organelles.<sup>[2,27,43,44]</sup> Thus, minimal mutated mtDNA carryover is expected,<sup>[43,44]</sup> with several studies showing undetectable mutated mtDNA when first PBT is performed, and around 2% when second PBT is used.<sup>[2,37,43]</sup> Nevertheless, the reduced amount of cytoplasm can have deleterious consequences.<sup>[27]</sup>

Another advantage of this technique is the easy visualisation and manipulation, without chromosome loss because of the cellular membrane <sup>[2,43]</sup> and with minimal damage as polar bodies are separated from the oocyte.<sup>[27,44]</sup> As the second polar body contains only a haploid genome, removal of the maternal pronucleus of the recipient zygote would be required. Removing only one pronucleus is challenging,<sup>[27,43]</sup> so the zygote should be enucleated and again fertilised after introducing the second polar body's genome.<sup>[43]</sup>

If PBT can be successfully performed in parallel with other

NT techniques, the number of donor oocytes required may be reduced by half.<sup>[2,43]</sup>

Further studies are required to confirm whether the incidence of DNA mutations in polar bodies is identical to that of the sibling oocyte.<sup>[44]</sup>

#### e. Blastomere transfer

It is still unclear whether the transfer of a blastomere from an affected embryo into an enucleated healthy donor oocyte can successfully prevent mtDNA disease because an entire cell is fused to the recipient oocyte.<sup>[32,45]</sup> This may result in higher levels of heteroplasmy <sup>[32]</sup> and in poor developmental competence.<sup>[45]</sup>

#### **Genome editing**

Genome editing to prevent mtDNA disease transmission consists of removing mutated mtDNA of heteroplasmic cells using site-specific restriction endonucleases, such as clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated protein 9 system (CRISPR/Cas-9).<sup>[33]</sup> With this technique, donor oocytes are not required.<sup>[46]</sup>

This technique is less invasive than NT.<sup>[46]</sup> However, the mutation load remaining is higher than that obtained after NT or PGD <sup>[7]</sup> and the mtDNA copy number may be below the threshold necessary for embryonic implantation and development.<sup>[22,46]</sup>

There is a risk of cleavage of essential genes due to off-target editing, and therefore careful design of the guiding molecules is required.<sup>[47]</sup>

Recently, a new approach was proposed in which, instead of removing the mutated DNA, its sequence is altered. This base editing technique converts one base pair to another at a target locus without requiring double-stranded DNA breaks. It has shown a good efficiency and less off-target modifications than CRISPR/Cas-9.<sup>[48]</sup> This technique has not yet been used in mtDNA, but it may be a potential new method.

#### Conclusion

As there is still no treatment for diseases caused by mtDNA mutations, prevention is of major importance.

The techniques here described were tested in substantially different conditions, which makes it difficult to compare their results. There is therefore a need for further research with similar conditions for all the techniques, and also for research into side effects and long-term consequences of these techniques.

As ethical issues remain a limitation, boundaries should be defined to allow further research in this area, possibly allowing more studies on embryos and long-term follow up of children and subsequent generations but still avoiding the "slippery slope" feared by many.

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## Uncommon vulvar findings in children and adolescents referred to a tertiary center over 14 years: a retrospective study and review of the literature

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#### ABSTRACT

**Background and Purpose:** Vulvar complaints are particularly common among children and adolescents; the etiologies largely depend on patient age. Most clinicians are not familiar with the normal variations of the female external genitalia, and this results in many unnecessary referrals. The aim of this study was to describe the frequencies of uncommon vulvar findings (other than ambiguous genitalia or vulvovaginitis) in children and adolescents examined in a tertiary referral center over a 14-year period.

**Methods:** Records of girls (ranging from newborns up to the age of 18 years) who attended a tertiary Pediatric-Adolescent Gynecology and Reconstructive Surgery Center with vulvovag-inal complaints during the period 2004-2017 were studied retrospectively. Patient age, rea-son for referral, presenting complaints, key aspects of clinical examination, diagnosis and management were recorded for each patient. Patients with ambiguous genitalia or vulvo-vaginitis were excluded from the analysis.

**Results:** A total of 67 females (37 adolescents and 30 children) were included in the study. Labial minora hypertrophy and labial adhesions were the most common findings in 18 (26.9%) and 17 (25.4%) of the cases, respectively. Less common diagnoses were genital warts in 7 (10.4%) patients, genital trauma in 5 (7.5%) patients, labia minora masses in 5 (7.5%) adolescents, and unilateral labial majora inflammation in 2 (3%) patients. Among the adolescents with labia minora masses, three were diagnosed with vascular malfor-mations, one patient underwent cyst removal, and one suffered labial minora inflammation. Other rare diagnoses included stenosis of the vaginal opening secondary to lichen sclerosus in 1 (1.5%) adolescent and extensive unilateral hemangioma of the vulva in 1 (1.5%) 15-month-old child. Finally, in 11 (16.4%) girls, no pathology was identified.

**Conclusions:** Comprehensive external genitalia examination is an important part of periodic health checks in girls (children and adolescents), given that there are some, albeit relatively few, genital findings that require immediate referral to a child and adolescent gynecologist in order to ensure timely intervention and the best possible long-term outcome for the young girl.

#### **KEYWORDS**

Vulva, labial adhesions, labial hypertrophy, genital warts, straddle injury, vascular malfor-mation, lichen sclerosus.

#### Introduction

Examination of the external genitalia is an important part of periodic health checks in female patients, starting from the neonatal period until adulthood. There are significant morphological differences in the external genitalia of prepubertal girls compared with adolescents. It is important for the clinician to be familiar with these differences, as well as with their physiological variations, in order to avoid unnecessary referrals to specialized centers, which place a significant burden on health systems and also cause anxiety, both to the young girl and her parents. However, it is also important that any suspicious finding is not missed and promptly referred to a specialized center.

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In childhood, vulvovaginitis is the most common reason for referral to pediatric gynecology clinics, with rates as high as 80% <sup>[1]</sup>. Other conditions which might present with similar symptoms include labial adhesions, lichen sclerosus, urethral



meatus prolapse, ulcers and straddle injuries<sup>[2]</sup>. In adolescence, external genitalia symptoms are usually expressed in the context of vulvovaginal infections, including sexually transmitted infections. Adolescents may also seek medical advice if they are concerned about the appearance of their external genitalia<sup>[3]</sup>. The aim of this study was to describe the frequencies of uncommon clinical findings of the external genitalia in prepubertal and adolescent girls presenting with vulvovaginal complaints (excluding ambiguous genitalia or vulvovaginitis) at a tertiary pediatric–adolescent gynecology and reconstructive surgery department over a period of 14 years.

#### Methods

This is a retrospective study of patients who presented with vulvovaginal complaints at the Pediatric–Adolescent Gynecology and Reconstructive Surgery Clinic of the 2<sup>nd</sup> Department of Obstetrics and Gynecology of the National and Kapodistrian University of Athens, based at the Aretaieion Hospital in Athens, Greece. Records of the period from 2004 to 2017 were retrieved and data were extracted for analysis. Study participants included females from birth up to the age of 18 years. Patient age, reason for referral, presenting complaints, key aspects of the clinical examination, diagnosis and management were recorded for each patient. Patients with a diagnosis of ambiguous genitalia or vulvovaginitis were excluded from the analysis. Images of external genitalia were also retrieved, and parental permission for their publication was obtained.

#### Results

The records of a total of 3250 patients were reviewed. Among them, 67 patients (age range 13 months – 18 years) were identified as having vulvovaginal symptoms. Patients were divided into 30 children (aged 13 months to 9.5 years) and 37 adolescents (aged 8.5 to 18 years) depending on their pubertal development (Table 1). Labial minora hypertrophy and labial adhesions were the most common findings.

Table 1 Vulvar findings in children and adolescents.

Labial adhesions of varying degree (35% to 95% of labiallength) were identified in 17 (25.4%) cases (Figure 1). In the vast majority (94.1%) this condition was observed in prepubertal girls (mean age ± SD, 4 ± 2.3 years); indeed, it was observed in only one adolescent, aged 13 years. Symptoms of vulvovaginitis were reported by 4 (23.5%) of these patients, with cultures from a high vaginal swab revealing mostly gut flora. In symptomatic patients, labial adhesions were bluntly separated in the clinic, with the aid of the "pull-down maneuver", after local application of lidocaine/prilocaine cream. In order to avoid recurrences, parents were instructed to ensure daily application of a water-based lube and proper local hygiene. Re-evaluation at 6 months post-treatment revealed recurrence of labial adhesions in 3 patients (17.6%), but to a lesser extent (10% to 30% of labial length).

Reported "deformities of external genitalia" was the reason for referral in 18 (26.9%) adolescents with a mean ( $\pm$  SD)

Figure 1 Extensive labial adhesions in a toddler.



	CHILDREN - N = 30 Age (Range) 13 Months – 9.5 yrs	ADOLESCENTS - N = 37 Age (RANGE) 8.5 – 18 yrs	TOTAL SAMPLE - N = 67 Age (Range) 13 Months – 18 yrs
Labial adhesions	16	1	17
Minor labial hypertrophy	-	18	18
Labial hematoma (injury)	3	2	5
Genital warts	5	2	7
Vascular malformations	-	3	3
Cyst of the minor labia	-	1	1
Labial minora inflammation	-	1	1
Labial majora inflammation	-	2	2
Lichen sclerosus	-	1	1
Hemangioma	1	-	1
No pathology identified	5	6	11

age of  $14.6 \pm 2.8$  years. All these referrals were cases of labial minora hypertrophy i.e. a labial minora stretched length of more than 4 cm, either unilateral or bilateral (Figure 2a). Although the adolescents and their parents were informed about the benign nature of this condition, four adolescents requested to undergo labiaplasty, which was performed after the age of 18 years (Figure 2b).

Vulvar and perianal genital warts were identified in 7 (10.4%) patients, 5 of whom were toddlers and prepubertal girls without any evidence of sexual abuse (age range 22 months to 4.2 years); the other two were sexually active adolescents (Figure 3). Two of the children were treated with cryotherapy and three with cauterisation, while the adolescents received im-

Figure 2a Labial minora hypertrophy in a 18-year-old adolescent girl.



Figure 2b External genitalia of the same patient after labiaplasty.



iquimod cream. Accidental injuries were the reason for urgent referral in 5 (7.5%) females (age range 3 to 13 years). Careful history taking revealed straddle injuries, while clinical evaluation revealed, in all cases, unilateral vulvar hematomas with localised edema without hymenal lacerations. All patients were managed conservatively.

Five (7.5%) adolescents complained of labia minora masses. One of these patients was found to have a simple cyst, one had a labial mass related to local inflammation, while three adolescents had vascular malformations of the vulva. In the latter, further ultrasound investigation revealed venous (Figure 4) and lymphatic vessel malformations (Figure 5) in two cases and one case respectively. Conservative management with regular

Figure 3 Genital warts in the vulva of a prepubertal girl.



Figure 4 Venous vascular malformation in external genitalia of an obese 12-year-old adolescent girl.



Figure 5 Lymphatic vascular malformation of the vulva (labia minora) in an 18-year-old adolescent girl.



follow-up was suggested.

Other rare cases included localized unilateral inflammation of the labia majora without evidence of Bartholin's gland abscess in 2 (3%) patients, one (1.5%) case of lichen sclerosusrelated vaginal introitus stenosis (Figure 6), and one (1.5%) case of extensive unilateral hemangioma of the vulva in a 15-month-old toddler.

Physical examination was normal in the remaining 11 (16.4%) cases; these patients, 5 pre-pubertal girls (aged 13 months to 5.8 years) and 6 adolescents (aged 10.5 to 18 years), had been referred with non-specific vulvovaginal complaints. In this group, minor labial size discordance was found in 3 patients, but it was nevertheless within the spectrum of normal variation.

#### Discussion

According to the findings of this 14-year retrospective study, "vulvovaginal complaints" were the presenting symptoms of a heterogeneous group of disorders (Tables 2 & 3). The most common finding in the prepubertal girls (average age

	LABIAL Adhesions	LABIAL MINORA Hypertrophy	GENITAL TRAUMA	GENITAL WARTS	VASCULAR Malformation	LICHEN SCLEROSUS	LABIAL INFLAMMATION
Asymptomatic	+	+		+	+		
Vaginal secretions	+					+	
Vulvar pain		+	+		+	+	+
Vulvar hematoma/ bleeding			+	+	+	+	
Pruritus				+		+	
Edema			+		+		+
Non-specific vulvar complaints	+	+			+		

Table 2 Common symptoms of vulvar pathology.

#### Figure 6 Lichen sclerosus-related vaginal introitus stenosis.



4 years) was the presence of labial adhesions. In the current literature, labial adhesions have been reported at ages ranging from 3 months to 6 years, with the peak incidence found to occur at the age of 2 years<sup>[4]</sup>. Rarely, labial adhesions present for the first time after the age of 6 years or persist in puberty. Girls with labial adhesions may be asymptomatic and adhesions can be detected incidentally by parents or pediatricians during a physical examination. Very occasionally, the vaginal orifice is completely covered, causing postvoid dripping of urine or vaginal secretions or non-specific vulvar complaints. In cases of recurrent uninary tract infections or vulvovaginitis treatment is required <sup>[5]</sup>. In the present study vulvovaginitis complicated about 1 in 4 cases of labial adhesions.

The exact causes of labial agglutination have not yet been clarified. The condition has been associated with low estrogen levels prepubertally, while in newborns the effect of maternal estrogens seems to exert a protective action <sup>[4]</sup>. Contributing factors include topical irritating agents, vulvovaginitis and minor local injuries. It has been suggested that sexual abuse might predispose to labial agglutination, but this theory has been challenged as the presence of other signs has been more robustly associated with abuse <sup>[6,7]</sup>.

#### Table 3 Key features of clinical examination.

VULVAR DISEASE	CLINICAL FINDINGS			
Labial adhesions	Erythema, a thin, pale membrane between labia minora partly covering the vaginal introitus, urine retention, vaginal secretions, normal labia majora			
Labial minora hypertrophy	Enlargement of one or both labia minora			
Genital trauma	Ecchymoses, abrasions, lacerations, hematoma $\pm$ trauma of hymen, vagina, anus, rectum			
Genital warts	Single or multiple, small, flesh-colored or hyperpigmented papules or plaques in the perianal or genital region, bleeding			
Venous vascular malformation	Asymmetric labia majora, soft, non-pulsating, painless mass, enlargement with Valsava maneuver			
Lichen sclerosis	White, atrophic, parchment-like skin, chronic ulceration, inflammation, subepithelial hemorrhages, scarring of the clitoral hood, thickening of the posterior fourchette, bleeding, hourglass configuration			
Labial inflammation	Erythema, edema $\pm$ vaginal discharge			

Diagnosis of labial adhesions can be based on the observation, on visual inspection, of a thin, pale membrane between the labia minora partially or even entirely covering the vaginal introitus. Differential diagnosis includes hymenal atresia, complete transverse septum of the lower vagina, Mayer-Rokitansky-Küster-Hauser syndrome and complete androgen insensitivity syndrome.

Spontaneous separation of labial adhesions has been reported in up to 80% of cases, especially in the case of small adhesions at the posterior fourchette and following estrogenization in puberty [5,8]. Adhesiolysis is recommended when labial fusion is symptomatic. This can be easily performed in the physician's office with gentle traction or with the use of a thin cotton swab, usually after application of local anesthetic gel. In rare cases of dense adhesions, lysis can be performed in hospital under regional or general anesthesia. Irrespective of the method used for treatment, it is essential to instruct parents to ensure adequate local hygiene, and to prevent adhesion recurrence by frequently separating the labia minora and by regularly applying a water-based lubricant gel for 6 to 12 months. Parents can be instructed on labial separation technique i.e. the use of two index fingers (one on each side) to gently pull down and push outwards the labia majora.

Local application of estrogen cream is another method for treating labial agglutination with success rates ranging from 50% to 88% <sup>[9-13]</sup>. Side effects of estrogen creams include breast budding, local discoloration and irritation of the skin and, more rarely, vaginal spotting or bleeding <sup>[9]</sup>. All the aforementioned symptoms subside after treatment discontinuation. Success rates of up to 70% have been reported after local treatment with betamethasone ointment, with commonest side effects being localized erythema, atrophic dermatitis and folliculitis <sup>[14, 15]</sup>.

Recurrence rates of labial adhesions are estimated to be between 11.6 % and 14%, or even as high as 17.6% in studies with longer follow-up <sup>[16]</sup>. Local application of estrogen cream after adhesiolysis is considered to be protective <sup>[9, 11, 16]</sup>.

Labia minora hypertrophy is defined as labial tissue protrusion beyond the labia majora. It can be either unilateral or bilateral. In this study labial hypertrophy was found in almost half of the adolescent patients referred to our center.

There is no agreement on a cut-off value of "normal" labia minora size. Friedrich *et al.* <sup>[17]</sup> suggested a cut-off value of 5 cm for the maximal length from midline to the lateral edge of

the labia minora. More recent studies have proposed smaller sizes of 3 to 4 cm [18-20]. However, in many cases, simple measurement of labia minora length is not sufficient to diagnose hypertrophy and other factors should be taken into account. Since there is no international consensus on normal size values, in the present study the criteria for a diagnosis of labial hypertrophy were a labial length >4cm in an adolescent with subjective symptomatology. Symptoms may include psychological distress, irritation (especially with activity), painful intercourse, and discomfort from tight fitting clothes [21]. In this study four adolescents underwent labiaplasty due to increased stress associated with their genital appearance. In a UK-based study of 33 women, this was the commonest reason for requesting surgical management [22]; while only two of these women had labia longer than 4 cm, five had labia measuring 4 cm and 26 women had a labial length of between 1 and 3 cm.

The cause of labial hypertrophy remains unknown, but it has been associated with genetic factors and elevated levels of estrogen and inflammatory factors, as well as with chronic mechanical irritation <sup>[23]</sup>. Masturbation had also been proposed as a potential contributing factor, but this theory has been disputed for several decades <sup>[24]</sup>.

The initial approach to asymptomatic patients should be conservative, and counseling should include reassurance that hypertrophic or asymmetrical labia constitute normal variations of external genitalia anatomy, growth and development. Surgical treatment should be considered after the age of 18 years in adolescents with severe functional symptoms and psychosocial distress. According to the Committee on Adolescent Health Care of the American College of Obstetricians and Gynecologists <sup>[25]</sup>, surgical correction in girls younger than 18 years should be considered only in those with significant congenital malformation or persistent symptoms that the physician believes are caused directly by labial anatomy, or both. Labiaplasty involves careful excision of excessive labial tissue, with great care being taken to maintain symmetry. Early complications include hematoma, edema and infection. Longterm complications include scarring, local hypoesthesia or chronic vulvar pain and. Dissatisfaction with the esthetic outcome has also been commonly reported.

Genital warts or condylomata acuminata are skin lesions caused by human papillomavirus (HPV) infection that typically appear as fleshcolored or hyperpigmented papules or plaques in the perianal or genital region. In adults they are associated with the HPV types 6 and 11 in 90% of cases. Other HPV types have also been implicated, including types 16, 18, 31, 33 and 35, usually in combination with types 6 and 11<sup>[26]</sup>. The HPV types detected in lesions from children are more variable and, as well as the aforementioned types, they may include types 1, 2, 4, 7, 27, 57, 60 and 63. In infants and toddlers up to the age of 2 years, the perianal region is the commonest lesion site, while in older children warts can be found in the vulva, vagina and periurethral region. The transmission route in this age group is uncertain, and although genital warts were considered to be a sign of sexual abuse, it is now believed that HPV contamination can happen through non-sexual routes i.e. during labour, self-inoculation, [27-33] etc. In these studies, [27, 29, 32] prepubertal girls with HPV infection had no evidence of sexual abuse and the exact route of HPV transmission could not be identified. Two of these girls (aged 2.5 and 4 years) had perianal warts, while the rest had warts on the perineum and the vulva.

Epidemiological data on childhood warts are scarce. The mean age of wart appearance ranges from 2.8 to 5.6 years <sup>[34]</sup>. According to a CDC report, 18.3% of sexually active adolescents aged 14 to 19 years have acquired at least one of the 23 HPV types associated with genital warts <sup>[35]</sup>.

In most cases, warts are incidental findings during pediatric examination. Symptoms are rare and vary from an itching or burning sensation to accidental bleeding. Spontaneous remission rates can be as high as 35% within 4 months, rising to 50%in 12 months and 90% after 24 months, thus making treatment during diagnosis unnecessary [36]. Available treatment regimens aim to obtain localized lesion destruction (through cauterization, cryotherapy, surgical excision or topical application of antimitotic agents). There are studies reporting satisfactory results with local application of imiquimod cream, but this treatment is not officially approved for use in children and adolescents [37]. All the aforementioned methods are associated with localized side effects, including erythema, edema, skin abrasion or symptoms such as pain, pruritus and burning sensation that may reduce compliance in this age group <sup>[37]</sup>. Considering all the above, it is recommended that treatment should be individualized and reserved only for persisting lesions or severe symptoms [26, 38-40]. The risk of HPV transmission is considered to be minimized after treatment, but since it cannot be eliminated, counseling should also include sexual and non-sexual protection education as well as promotion of anti-HPV vaccination especially in populations with low vaccination uptake<sup>[26]</sup>.

Vascular anomalies, according to the updated classification of the International Society for the Study of Vascular Anomalies, have been classified in two groups, vascular tumors and vascular malformations <sup>[41]</sup>. Hemangiomas are the commonest benign vascular tumors in infancy caused by the rapid proliferation of vascular endothelial cells <sup>[42]</sup>. Risk factors include white ethnic origin, female sex, prematurity and prenatal testing with chorionic villus sampling. Hemangiomas of the vulva tend to be superficial and of limited size and can be associated with congenital anomalies of the urogenital tract or of the perianal region. Lesions of the vaginal wall and the perineum are prone to abrasion and infection due to mechanical irritation <sup>[43]</sup>. In most cases, diagnosis is made by clinical evaluation. In cases with multiple skin hemangiomas it is essential to offer ultrasound examination of abdominal organs. Treatment should be reserved for symptomatic cases when functional problems or bleeding occur. Ulcers should be treated with local application of antibiotics combined with corticosteroids, hydrating creams and adequate analgesia <sup>[11]</sup>. Systematic administration of corticosteroids can inhibit the growth or even contribute to the involution of hemangiomas. The use of the betablocker propranolol has also been shown to be beneficial, but there are no standardized treatment protocols yet <sup>[44]</sup>. Other treatment methods that have been proposed include interferon, vincristine, embolization and laser treatment.

Vascular malformations are considered to result from developmental errors during embryogenesis which lead to the persistence of vascular plexus cells with a certain degree of differentiation. They are divided into 4 groups i.e. simple malformations, combined malformations, malformations of major named vessels, and malformations associated with other anomalies. Simple malformations are further categorized, according to the prevailing vessel type, into capillary, lymphatic, venous or arteriovenous malformations and arteriovenous fistulas <sup>[41]</sup>. On the basis of their flow characteristics they are further grouped into low flow (capillary, lymphatic and venous) and high flow (arterial/arteriovenous malformations and arteriovenous fistulas) velocity [45]. The exact prevalence of vascular malformations remains unknown, due to the fact that classification systems have changed over time. Venous malformations seem to be the commonest lesions and comprise about 2/3 of total cases.

Venous malformations can be visible after birth and tend to increase in size as the infant grows or can become apparent for the first time during adult life. The increased prevalence in females as well as their rapid growth during puberty and pregnancy suggest that the hormonal milieu plays a significant role in their pathophysiology <sup>[46-48]</sup>. They are more frequently located in the perineum and the labia majora and tend to become more apparent after periods of prolonged standing, walking or intense exercise, with concomitant intensification of symptoms like edema, pain and localized pressure [49]. Clinical examination is sufficient to establish the diagnosis. There is usually a visible soft, non-pulsating, painless mass that lends the labia majora an asymmetric appearance. Venous malformations tend to increase in size with the Valsava maneuver. Differential diagnosis includes haematomas, varices, neoplasms and other vascular malformations.

Lymphatic malformations consist of dilated lymphatic channels or cysts, lined with lymphatic endothelial cells. They are classified as microcystic, macrocystic and mixed subtypes. The most common sites are the head and the upper limbs, and rarely the female genital tract. They can be associated with venous or capillary malformations. Symptoms include intermittent pain and edema, infections and hemorrhage; they can also cause appearance dissatisfaction.

Investigations for the diagnosis of vascular malformations include Doppler sonography and magnetic resonance imaging <sup>[50]</sup>. Treatment is typespecific, but also depends on lesion location and flow characteristics. Most of the vulvar dysplasias can be managed with the use of sclerotherapy or surgical excision. In the abovecited study, <sup>[50]</sup> all patients were successfully managed conservatively.

Lichen sclerosus is a complex chronic inflammatory skin condition which mainly manifests itself in the vulvar area of preadolescent and adolescent females [51]. The pathophysiology of lichen sclerosus is not clearly understood; it is considered an autoimmune disease with genetic component as suggested by studies in monozygotic twins [52]. The most common symptoms are pruritus, dryness, dysuria and hemorrhage, while bowel-related symptoms and abnormal vaginal discharge have also been reported. Clinical examination reveals a whitish fragile atrophic lesion with a cigarette paperlike appearance. Signs of chronic ulceration, inflammation and subcutaneous hemorrhage can also exist. Progression to more severe disease stages causes disruption of the normal vulvar anatomy with formation of scar tissue over the clitoris and the labia minora as well as thickening of the labial posterior fourchette. Involvement of the perineum and the perianal area along with the labia may give the affected area an hourglass configuration. Diagnosis is based on the typical appearance and biopsies are rarely required.

Mild cases can be managed conservatively through application of hydrating creams, improvement of hygiene and avoidance of irritating factors. Should these prove to be inadequate, local corticosteroids can be applied until complete remission of symptoms. Recurrences are common and are also treated with topical corticosteroids. Surgical intervention may be needed later if loss of vulvar architecture and adhesions around the clitoris develop. After the onset of pubertal development, the effect of estrogens may aid disease remission <sup>[53, 54]</sup>.

Trauma in the genital area in childhood and adolescence can result from an isolated injury (e.g. straddle injury) or from multiple injuries (e.g. after a car accident). When there is a marked discrepancy between findings and the possible mechanism of injury, sexual abuse should always be part of the differential diagnosis [55]. A study [56] of 358 cases demonstrated an increased prevalence of genital trauma in those aged under 10 years. In particular, trauma, as a result of sexual abuse, occurred more frequently in infants and toddlers up to the age of 4 years, falls or bicycle accidents were more prevalent in the ages between 5 and 9 years, whereas car accidents were more frequent in adolescents older than 15 years. It has been hypothesized that perineal and genital area tissues are more fragile in the preadolescent years. Perineal trauma accounts for almost 0.2% of all traumas in girls younger than 15 years <sup>[57]</sup>. Clinical findings include bruises, lacerations, tears and hematomas.

About 15-20% of reported cases required surgical treatment, while in the absence of sharp object injury, this risk drops to 9% <sup>[56, 58, 59]</sup>. Conservative management includes reduced activity, sitz baths and adequate analgesia, especially within the first 24 to 48 hours <sup>[58]</sup>.

In children under the age of 14 years, straddle injuries are the most common cause of genital trauma that occurs as a result of tissue compression between a hard object (bicycle saddle, seesaw, home furniture) and the bony pelvis. In the present study all cases of genital traumas were caused by straddle injuries, with labial hematomas being the most common finding. One case presented with introital hematoma and another one with marked edema of the hymen without evidence of rupture. Generally, the genital areas that are more commonly affected by non-penetrating injuries are the labia majora, the pubic area and the clitoris, while the vagina and the hymen usually remain intact.

Other genital injuries reported in the literature include accidental penetrating injuries, vaginal trauma caused by extreme water pressure (e.g. jet-ski accidents), trauma caused by fractured pelvic bones, bites, burns and penetrating trauma during intercourse (consensual or non-consensual). A special mention should also be made of female genital mutilation, which is still performed in various regions around the world <sup>[60]</sup>.

#### Conclusions

External genitalia problems can be a source of distress for both the young girl and her parents. In most cases, though, they are associated with benign conditions which require either no or only minimal intervention. In every case, evaluation by child and adolescent gynecologists or other specialized clinicians, familiar with the rare conditions and the physiological variations of the female genital area, is warranted in order to ensure the best possible longterm outcome.

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# Laparoscopic treatment of borderline ovarian tumours. A systematic review of the literature

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#### ABSTRACT

**Background and purpose:** The laparoscopic management of borderline ovarian tumours (BOTs) is controversial. The aim of our study was to review the scientific literature on this approach.

**Methods:** The search strategies used included an online search of the MEDLINE database of relevant publications and reviews from 2003 to 2018 regarding laparoscopic treatment of BOTs. Additional reports were collected by systematically reviewing all the references from the retrieved papers.

**Results:** Recent articles have discussed the type of surgery (laparotomy or laparoscopy), the possibility of fertility-sparing surgery, and the need for restaging procedures and adjuvant therapy.

**Conclusions:** Over recent decades, the management of BOTs has shifted from radical surgery to more conservative therapy due to the need for fertility-sparing surgery and the increasing use of laparoscopy.

#### **KEYWORDS**

Borderline ovarian tumour, borderline ovarian neoplasm, atypical proliferative tumour, surgery, laparoscopic treatment, laparoscopic management, minimal invasive surgery.

#### Introduction

Borderline ovarian tumours (BOTs) are uncommon but not rare ovarian neoplasms. Their incidence is low, with around 4.8/100 000 new cases per year calculated in European series<sup>[1]</sup>; the incidence is even lower in American series: between 1.5 and 2.5/100 000 cases per year <sup>[2,3]</sup>.

BOTs occur in women at approximately 40 years of age (or at a younger age in 27-36% of cases), as opposed to an average age of 60 years in the case of invasive carcinoma<sup>[4]</sup>; BOTs are also defined as ovarian tumours with low malignant potential. These tumours account for 15% of all epithelial ovarian cancers and are not BRCA related. More than 80% of women with BOTs present with stage I disease. As indicated above BOTs, are neoplasms of epithelial origin, and constitute an intermediate category between benign and malignant froms <sup>[5]</sup>; they are characterised by upregulated cell proliferation and the presence of slight nuclear atypia, but without destructive stromal invasion <sup>[5]</sup>. The current 2014 World Health Organisation (WHO) Classification of Tumours of the Female Genital Organs uses the term "borderline tumour" interchangeably with "atypical proliferative tumour"<sup>[6]</sup>. The 5- and 10-year survival rates for early-stage BOT (stage I) are 99 and 97%, respectively, and thus, conservative treatment is an option. Nevertheless, survival rates are less favourable for advanced stages of BOT, especially in the presence of invasive implants, and alternative treatment options need to be explored to preserve fertility in these patients <sup>[7]</sup>.

Among BOTs, six histological subtypes can be classified. On the basis of the epithelial cell type, serous (50-55%), mu-

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cinous (35–45%), endometrioid (2–3%), clear cell (<1%), seromucinous (5–7%) and borderline Brenner tumour (3–5%) subtypes can be distinguished <sup>[8]</sup>. Mucinous BOTs are classified as intestinal (85%) or endocervical/Mullerian type (15%), depending on the nature of the epithelial lining. They can be associated with pseudomyxoma peritonei (10%), necessitating a thorough investigation of the gastrointestinal tract with special attention to the appendix, which can be the primary tumour origin. BOTs can show microinvasion, lymph node and peritoneal spread, critically influencing the disease prognosis <sup>[9, 10]</sup>. Ovarian serous borderline tumours (SBTs) have been the subject of considerable controversy, especially with regard to terminology and behaviour.

It has been proposed that they constitute a heterogeneous group of tumours, mostly comprising typical SBTs that are benign and designated atypical proliferative serous tumours (APSTs) and a small subset of SBTs with a micropapillary architecture that have a poor outcome and are designated non-invasive low-grade serous carcinomas (niLGSCs). It has also been argued that the difference in behaviour between the two groups is not due to the primary tumour subtype, but rather to the presence of extra-ovarian disease, specifically invasive implants <sup>[11]</sup>. Among patients with stage I disease, the risk of subsequent serous carcinoma was significantly higher in those with niLGSCs as opposed to APSTs. Nonetheless, all-cause mortality risk is not different between APSTs and niLGSCs, among either stage I or >I cases. In addition, although the presence of invasive implants is the single most adverse prognostic factor, sub-classification into APSTs and niLGSCs is important because it allows stratification of stage I cases in terms of risk of advanced stage disease and invasive implants and subsequent development of serous carcinoma. Finally, it is important to emphasise that, although invasive implants carry the highest risk of subsequent serous carcinoma and all-cause mortality, non-invasive implants are also associated with a statistically significantly increased risk - albeit not nearly as high as that recorded for invasive implants <sup>[12]</sup>.

This review aims to provide a comprehensive and systematic review on laparoscopic treatment of BOTs analysing reviews, observational, cohort and case-control studies on this surgical approach that offer great advantages both for patients and for the healthcare system <sup>[13,14]</sup>.

#### Methods

We searched the PubMed and MEDLINE electronic databases to find relevant articles published in the period 2003 to 2018. The search strategy was based on the following terms: "borderline ovarian tumour", "borderline ovarian neoplasm", "atypical proliferative tumour", "surgery", "laparoscopic treatment", "laparoscopic management" and "minimally invasive surgery". This review covers both early and advanced stages of BOTs, as well as rare entities like endometrioid, Brenner and clear-cell BOTs. All case reports, original studies, meta-analyses and reviews published in English and French were considered. In the case of duplicate publications from the same team, the most recent study was included.

Relevant studies were evaluated by all the authors, and a consensus decision was made on their eligibility for inclusion in this review.

#### Results

The electronic database literature identified 2351 articles about BOTs: 769 articles dealing with the physiopathology and diagnosis of BOTs and 1582 with BOT management. Of these, 1062 focused on non-surgical treatment and 520 on surgical treatment; of the latter, 448 articles dealt with laparotomy surgery, and just 72 with laparoscopic treatment of BOTs and laparoscopic versus laparotomy treatment; these 72 articles were included in this review (Figure 1).

Fertility is frequently an issue when discussing treatment options <sup>[15-18]</sup>. However, an equally important issue is whether we can reduce the morbidity caused by radical surgery and whether a more conservative approach is a safe alternative in terms of the cancer prognosis.





#### **Radical surgery**

In postmenopausal women with BOTs, as well as in patients who have fulfilled their reproductive desire, radical surgery may be suggested. This generally involves bilateral salpingo-oophorectomy, total hysterectomy, inframesocolic omentectomy, peritoneal lavage to obtain samples for cytology, resection of macroscopically suspicious lesions and multiple peritoneal biopsies (including the omentum, intestinal serosa, mesentery, pelvic and abdominal peritoneum)<sup>[19]</sup>.

In the case of mucinous ovarian tumour identified on histological examination, especially in the context of pseudomyxoma peritonei, appendectomy should be performed to exclude a mucinous neoplasm of the appendix [19]. The role of retroperitoneal restaging remains unclear in the context of BOTs; it does not seem to be as crucial in these tumours as in their malignant counterparts, in which it may have both prognostic and therapeutic implications <sup>[20,21]</sup>. The results of a study conducted by Seidman and Kurman<sup>[9]</sup> suggested that systematic lymphadenectomy can be omitted from the initial treatment plan for BOTs; these researchers observed a survival rate of 98% among 43 women with nodal involvement, after a mean follow up of 6.5 years. Laparoscopy can be used successfully for performing a correct radical surgery in patients with BOTs. In two studies, the type of surgical approach (laparoscopic vs laparotomy) did not appear to influence the progression-free interval and the rate of recurrence <sup>[22,23]</sup>. Recent studies have also shown that laparoscopic surgical staging of ovarian cancer at an early stage is just as safe and adequate as laparotomy staging [24].

#### **Conservative surgery**

A conservative treatment may be proposed for women under the age of 40 years who have not completed childbearing <sup>[25]</sup>. In these cases, oophorectomy, unilateral salpingo-oophorectomy or cystectomy may be performed. Exploration of the cavity, omentectomy, peritoneal washing, resection of suspicious lesions, multiple peritoneal biopsies and adnexectomy are recommended, as for radical surgery, in the case of mucinous BOTs [26]. In a systematic review of the conservative management of BOTs, Darai et al. [7] showed that the rate of relapse after conservative treatment is 0-25%. This rate is higher than the rates reported for conventional radical surgery by bilateral salpingo-oophorectomy with or without hysterectomy, which vary between 0 and 5%. The rate of recurrence is correlated with the type of conservative treatment used (salpingo-oophorectomy or cystectomy), with a higher rate of 10-42% recorded in patients undergoing cystectomy. Moreover, in a randomised trial, Palomba et al. [27] reported a time to recurrence of 16 months in patients undergoing bilateral cystectomy versus 48 months in patients undergoing unilateral salpingo-oophorectomy and contralateral cystectomy. In this study, 32 patients with bilateral BOTs, treated laparoscopically, were randomised to bilateral cystectomy or unilateral salpingo-oophorectomy on the greater lesion and contralateral cystectomy. The cumulative pregnancy rate and cumulative probability of a first pregnancy were higher in patients treated with bilateral cystectomy. Nonetheless, patients undergoing bilateral cystectomy had a shorter time to first recurrence and a higher rate of radical treatment of the recurrence. These results suggest that bilateral cystectomy should be performed in the case of bilateral serous BOT if technically feasible in motivated patients to improve fertility but after informed consent on recurrences. Unilateral cystectomy is probably a better option for improving fertility in cases of unilateral tumour. According to du Bois et al. [28], when conservative surgery was performed via laparotomy, the recurrence rate was about 7.7%, while it was as high as 14.9% after laparoscopy. However, in a multicentre study, there were no significant differences in recurrence rates after laparoscopy versus open surgery <sup>[23]</sup>. In their series of 687 patients with BOTs, Song et al. showed that laparoscopy and open surgery were both feasible in cases of small-volume disease and the absence of peritoneally disseminated disease. However, the laparoscopic approach was associated with more favourable surgical outcomes, including decreased operative time, operative blood loss and transfusion rates, as well as faster bowel movement recovery, shorter postoperative hospital stays and fewer perioperative complications, with no compromise in oncological outcomes <sup>[29]</sup>. The equivalent and much larger German study (ROBOT) showed that, compared with open surgery, initial laparotomy and laparoscopy converted to laparotomy showed hazard ratios of 1.176 (0.772–1.792) and 1.213 (0.582–2.527), respectively, indicating that laparoscopy is comparable to open surgery in terms of oncological outcomes, relapse rate and overall survival [30]. For these reasons, the proportion of early-stage gynaecological cancers managed with minimally invasive surgery has increased from 7% to 90% [31]. Although it is difficult to reach definitive conclusions on this matter due to the lack of prospective studies, a skilled gynaecological oncologist with sufficient experience is best suited to perform laparoscopy surgery on BOT patients. In a retrospective French multicentre study of 358 patients, Fauvet et al. [22] confirmed that cyst rupture (33.9% vs 12.4%) and incomplete staging occurred significantly more frequently in the laparoscopy group. However, this had no influence on the relapse rate. The potentially higher risk of relapse and possible need for repeated surgery in this case, albeit commonly with no survival difference, should be discussed with the patient when balancing weighing up cosmesis and surgical burden. Routine biopsy on the contralateral ovary and multiple peritoneal biopsies are not considered necessary unless an abnormality appears macroscopically, since such procedures increase the risk of postoperative adhesions and are not of high value diagnostically because they may not produce a tumour sample [26].

Cystectomy, which produces an increased risk of recurrence on the ipsilateral ovary [32], should be carried out only in women with bilateral tumours or only one ovary, as well as extremely young patients. The increased relapse rate after cystectomy may be caused by the following: intraoperative cyst rupture, the presence of a multifocal BOT, or tumour margins affected after the cystectomy [33]. Nevertheless, most of these recurrences are borderline type, so they do not affect global survival rates [22,34]. From the analysis of the data in an Italian study <sup>[23]</sup>, it emerged that, considering the case series as a whole, the incidence of cyst rupture or spillage was effectively higher during laparoscopic surgery; nevertheless, on analysing the fertility-sparing surgery cases, no statistically significant difference in the incidence of spillage was found either when comparing laparoscopic versus laparotomy cystectomy, or laparoscopic versus laparotomy adnexectomy. Moreover, the relapse incidence did not depend on the type of surgical approach, even when considering only the group of cases where the spillage was caused by the surgeon. In addition, analysis of progression-free survival in these case series shows that only cystectomy has to be considered a risk factor. Invasive recurrent disease is a rare event after conservative treatment. Recurrent lesions of a non-invasive nature can be cured only by surgery, without affecting survival. It appears that the increased recurrence rate observed after conservative surgery does not influence survival [35]. One of the factors that increases the rate of recurrence after conservative treatment is the disease stage (especially in serous BOTs, where peritoneal implants can be found in 15-40% of cases). In a large series, Uzan et al. [36] demonstrated that conservative management may be an option for patients with peritoneal implants if the implants can be entirely removed. In mucinous BOTs, cystectomy is not recommended as a treatment for preserving fertility due to the high risk of recurrence in the form of carcinoma, as mucinous BOTs are globally associated with a higher mortality rate. For women under the age of 40 years who desire to have children and present with BOTs in stages II and III (with peritoneal implants), the surgical technique will vary according to the invasiveness of the implants: in patients with non-invasive implants, conservative surgery and total resectioning of the peritoneal implants may be carried out; in patients with invasive implants, radical surgery with complete resectioning of the implants is preferable [19].

#### Discussion

The surgical approach to BOTs is still under debate: while laparoscopy has become the standard approach for benign ovarian tumours, it has not been clearly proven to guarantee adequate staging or oncological safety in BOT patients [37-39]. The major concerns with laparoscopy are higher rates of cyst rupture, leading to a iatrogenic spread of tumour cells with a possible influence on patients' prognosis and clinical course <sup>[40]</sup>. Other disadvantages include a lack of tactile sensation, the development of port site metastases [41-43], and the problem of more difficult manipulation and removal of larger masses through the abdominal wall. The diameter of the cyst is a significant factor predicting laparoscopic failure, and it seems that laparoscopy should be reserved for ovarian masses of less than 5 cm [44]. In contrast, laparoscopy allows better illumination of the abdominal cavity, with better views of the peritoneal surface and diaphragm, lower risk of postoperative adhesions, better aesthetic results and quicker recovery times. In an original study by Delle Marchette et al., the surgical approach did not influence recurrence or fertility [45]. The main advantage of the mini-invasive approach is the reduced morbidity, especially the reduction of post-operative adhesions that could possibly impair fertility [46].

On the other hand, it is crucial that gynaecological oncologists have adequate laparoscopic experience in order to avoid cyst fluid spillage and limit healthy ovarian tissue ablation, thereby maximising the oncological and reproductive outcomes. The choice of surgical approach should be made taking into account on the size of the suspicious mass, the presence of adhesions, and the invasiveness of the surgery, while treating large cysts by laparoscopy should be avoided, as this enhances peritoneal tumour persistence and early relapse <sup>[45]</sup>. Most BOT patients are diagnosed in an early stage when the disease is still limited to the ovaries (78.5% in FIGO stage IA/B) <sup>[47]</sup>.

Proper staging consists of a thorough exploration of the entire abdominal cavity with peritoneal washing, infracolic omentectomy, removal of all macroscopically suspicious peritoneal lesions and multiple peritoneal biopsies. Complete staging is performed in only 50% of patients or less, even though the pelvic peritoneum and abdominal peritoneum are involved in 58% and 48% of patients, respectively. Furthermore, invasive implants are present in the pelvic peritoneum and abdominal peritoneum in 9% and 14% of patients, respectively. The omentum is involved in 39% of patients, and in 9% of patients, these implants are invasive. Hence, a careful inspection of the peritoneum with resection of macroscopically suspicious lesions, multiple peritoneal biopsies and an infracolic omentectomy are necessary for a thorough staging <sup>[48]</sup>. The role of lymph node sampling is controversial due to the good global prognosis of this disease and the potential morbidity associated with the procedure <sup>[49,50]</sup>. Routine pelvic and para-aortic lymph node dissection is unnecessary for most women with BOTs [51]. Accordingly, a study conducted at the European Institute of Oncology (IEO), showed that the restaging procedure does not seem to have a significant effect on the management of patients diagnosed with BOTs <sup>[52]</sup>. Although staging is sometimes less extensive with laparoscopy, the difference versus laparotomy falls short of statistical significance. In addition, laparoscopic surgery has not been associated with a deterioration in outcomes <sup>[29,53-56]</sup>.

#### Conclusion

Borderline tumours affect younger patients than invasive epithelial ovarian cancers do. Especially for young patients with early-stage borderline tumours who wish to preserve their fertility, the laparoscopic approach seems preferable <sup>[57-62]</sup>. Moreover, laparoscopic treatment can be practiced during pregnancy <sup>[63-65]</sup>. The risk of post-operative abdominal adhesions is considered to be lower after laparoscopy <sup>[46]</sup>.

Unilateral oophorectomy and omentectomy is considered to be a safe conservative surgery option for patients with BOTs <sup>[66]</sup>, provided the contralateral ovary is macroscopically normal. Ovarian resection or cystectomy should be avoided if the cyst looks suspicious on preoperative ultrasound examination, and the surgeon should try to avoid rupture of the cyst and intra-abdominal spillage <sup>[67,68]</sup>. Laparoscopic treatment of BOTs is feasible if the tumour is of moderate size, as this results in fewer complications and shorter hospital stays; furthermore, laparoscopic staging seems feasible if performed by experienced surgeons <sup>[7]</sup>.

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#### Adverse effect of higher waist circumference in assisted reproductive technology outcomes

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#### ABSTRACT

**Background and purpose:** Studies on the influence of obesity on the outcomes of treatment with assisted reproductive technologies (ART) are contradictory, although most have demonstrated a negative impact. The contribution of abdominal obesity, an effective marker of dysfunctional adipose tissue, has been poorly investigated.

**Methods:** Observational cohort study. All women (N=578) who underwent ART treatment at the fertility centre of a Portuguese University Hospital during the study period were included. The women's body mass index (BMI) and waist circumference (WC) were evaluated at the beginning of the stimulation cycle. They underwent controlled ovarian hyperstimulation with long agonist or short antagonist/agonist protocols. Data were stratified in two groups, according to the women's WC-based metabolic risk (defined according to Portuguese national guidelines): lower metabolic risk (WC < 88 cm) and higher metabolic risk (WC  $\geq$  88 cm).

**Results:** The women with a WC < 88 cm had a higher number of oocytes collected ( $\approx$  8% more, p=0.049), a higher number of mature oocytes ( $\approx$  20% more, p=0.010), a higher number of fertilized oocytes ( $\approx$  28 more, p=0.017) and a lower gonadotropin requirement ( $\approx$  10% less, p=0.042) than the women with a WC  $\geq$  88 cm. The chance of fertilization occurring was two times higher in women with a WC < 88 cm (OR [95% CI]:2.04 [1.04-4.00]). No significant associations were observed between WC group and pregnancy, live birth, cycle cancellation and miscarriage rates.

**Conclusions:** Women with a higher WC have poorer ART treatment outcomes, namely a lower number of oocytes collected, a lower number of mature oocytes, and a lower number of fertilized oocytes. This study highlights the importance of considering fat distribution in the quest to clarify the impact of obesity on ART treatment outcomes. According to our results, women with a WC  $\geq$  88 cm have poorer outcomes. It would be important to consider women's fat distribution when counseling and predicting ART treatment outcomes, particularly in terms of ovarian stimulation and oocyte quality.

#### **KEYWORDS**

Abdominal obesity, body mass index, assisted reproductive technologies, in vitro fertilization.

#### Introduction

Obesity is a worldwide epidemic and has more than doubled since 1980. In 2014, 39% of adults were overweight and 13% were obese <sup>[1]</sup>. Adipose tissue produces bioactive proteins, known as adipokines, which are involved in the coordination of several biological processes, including reproductive functions <sup>[2]</sup>. Excess adipose tissue is associated with ovulatory dysfunction <sup>[3]</sup>, reduced conception rates and longer time to conception <sup>[4-6]</sup>.

Studies on the influence of obesity on the outcomes of treatment with assisted reproductive technologies (ART treatment) are contradictory. Most studies have shown that body mass index (BMI) is directly associated with a higher gonadotropin requirement <sup>[7,9]</sup>; however, some studies have reported that obese women did not require significantly higher doses of gonadotropins <sup>[10-12]</sup>. Similarly, the number of oocytes collected and the number of mature oocytes were lower in the presence of raised BMI in some studies, <sup>[9-12]</sup> but not found to be affected by BMI in others <sup>[13,14]</sup>. Two meta-analyses evaluated the outcomes of ART treatment in obese and overweight women; both showed lower pregnancy rates among these women, although

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reached different conclusions regarding live birth rates: while Rittenberg *et al.*<sup>[15]</sup> found a relative risk of 0.84 for obese and overweight women, Maheshwari *et al.*<sup>[16]</sup> reported insufficient evidence on the effect of BMI on live birth. Another meta-analysis that assessed the effect of obesity and overweight on complications concluded that excess adipose tissue had an only slightly negative effect on ART outcomes, since there were no significant differences in complications, including ovarian hyperstimulation syndrome, ectopic and multiple pregnancy <sup>[17]</sup>. Metwally *et al.*, in their meta-analysis, reported that there is insufficient evidence to describe the effect of obesity on miscarriage rates after ART treatment <sup>[18]</sup>. Because of these conflicting results, the need to study other adiposity measurements



has been suggested.

The importance of adipose tissue distribution is emphasized in the definition criteria of metabolic syndrome. According to the joint statement released in 2009 by WHO, obesity is diagnosed using waist circumference (WC) and not BMI, as WC has been shown to better correlate with visceral adiposity and insulin resistance <sup>[19]</sup>. WC, after a certain threshold, increases the risk of cardiovascular disease and type 2 diabetes mellitus. The cut-off point for this threshold is population-specific and country-specific. Portuguese national guidelines state that women with a WC  $\geq$  88 cm have a very high metabolic risk <sup>[20]</sup>.

Visceral fat is thought to contribute to a greater amount of free fatty acids (FFAs) in the hepatic circulation, which may impair liver metabolism and contribute to insulin resistance <sup>[2]</sup>. Although insulin resistance is not included as a diagnostic feature, it has been reported in up to 50% of obese women with polycystic ovary syndrome, a condition characterized by oligo/anovulation, hyperandrogenism and polycystic ovaries [21]. Moreover, in an animal model of glucocorticoid-induced insulin resistance, dexamethasone was responsible for decreased circulating levels of estradiol and anovulation [22]. The expression of adipokines also differs according to the site of fat deposition, with a higher secretion of inflammatory cytokines and lower secretion of leptin and adiponectin in visceral adipose tissue <sup>[2]</sup>. Both of these adipokines are involved in the regulation of reproductive functions, including gonadotropin secretion and steroidogenesis (leptin) [23], oocyte maturation and early embryo development [23,24].

Wise *et al.* observed that obese women have a longer timeto-pregnancy and this association became stronger after controlling for WC. Notwithstanding, the relationship of WC and waist-to-hip *ratio* (WHR) with fecundability rate was null or weakly positive <sup>[6]</sup>. The few studies that have assessed fat distribution on ART treatment outcomes have found that WHR was inversely associated with the probability of conception per *in vitro* fertilization (IVF) cycle <sup>[25]</sup> and that a WHR  $\ge$  0.8 reduced the pregnancy rate of embryo transfer <sup>[26]</sup>.

Following the widespread clinical use of WC as a marker of abdominal obesity and metabolic risk, we searched for differences in ART treatment outcomes in women who, according to their WC measurement, presented different levels of metabolic risk.

#### **Materials and Methods**

#### 1. Study population

All the women who underwent ART treatment – *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) – at the Human Reproduction Department of a Portuguese University Hospital between October 2012 and August 2014 were included in the study. The women's BMI and WC were evaluated at the beginning of the stimulation cycle. The WC measurement was taken midway between the lowest rib and the iliac crest. Other clinical information, such as the women's age, the duration and cause of infertility, smoking habits, number of previous IVF cycles, ovarian stimulation protocol, type of fertilization (IVF or ICSI), and semen characteristics were recorded.

#### 2. Ovarian stimulation protocols, embryo transfer and follow-up

The women underwent controlled ovarian hyperstimulation (COH) with one of the following protocols: short antagonist protocol, long agonist protocol or short agonist protocol. A small percentage of women (5.2%) were stimulated with other combinations of drugs. The choice of protocol was made on a case-by-case basis according to patient clinical characteristics. COH was achieved by administration of recombinant follicle-stimulating hormone (FSHr) (Puregon®, Organon, Netherlands; or Gonal-F®, Merck Serono, Italy) or human menopausal gonadotropin (hMG) - Menopur®, Ferring, Germany. Doses ranged from 75 to 450 IU/day depending on the women's age, antral follicle count, FSH and anti-Müllerian hormone levels, and response to previous COH. From day 5 of stimulation, gonadotropin doses were adjusted according to serum oestradiol (E2) levels and ovarian response, assessed by vaginal ultrasound examination. In the short antagonist protocol, ovarian stimulation was initiated on day 2 of the menstrual cycle. Administration of a daily dose of 0.25 mg of gonadotropin releasing hormone antagonist (GnRHa) (Cetrotide®, Merck, Germany; or Orgalutran® Organon, Ireland) was initiated when the larger follicle reached a mean diameter of 14 mm. In the long agonist protocol, pituitary desensitization with daily subcutaneous administration of triptorelin 0.1 mg (Decapeptyl<sup>®</sup>, Ipsen Pharma Biotech, France) began in the midluteal phase of the previous menstrual cycle. This dose was continued until ovarian quiescence was confirmed by ultrasound examination and by E2 level (<50 pg/mL), at which point the dose of the GnRH was halved and maintained until ovulation induction in combination with FSHr or hMG. In the short agonist protocol, a daily dose of 0.1 mg of triptorelin (Decapeptyl®, Ipsen Pharma Biotech, France) was initiated at day 2 of the menstrual cycle in combination with gonadotropin administration. Ovulation and oocyte maturation were induced with intramuscular administration of 5000 or 10000 IU of human chorionic gonadotropin (hCG) (Pregnyl<sup>®</sup>, Organon, Netherlands) when at least three leading follicles reached a mean diameter of 17 mm. Transvaginal oocyte retrieval was scheduled 34 to 36 hours after hCG administration. Women were instructed to begin micronized intravaginal progesterone 200 mg every 8 hours (Progeffik<sup>®</sup>, EFIKK, France) from the day of the fertilization. Fertilization was assessed after 18 hours and fertilization rates were calculated. Embryo cleavage was assessed every 24 hours thereafter and transfer was performed 3 or 5 days after oocyte retrieval. Fourteen days after oocyte retrieval a quantitative serum value of β-hCG was obtained.

#### 3. Outcome measures

The outcome measures considered in this study were outcomes of assisted reproductive treatments, including dose of gonadotropin used for ovarian stimulation, number of oocytes collected, number of mature oocytes, number of cancelled cycles, embryo development, fertilization, implantation and miscarriage rates, and pregnancy and live birth rates.

Clinical pregnancy was ascertained by the presence of an intrauterine gestational sac on ultrasound examination and was expressed per cycle started as well as per embryo transfer. Live birth was considered achieved when the fetus was born alive beyond the 24th week of gestation. Embryo morphology (number of cells and degree of fragmentation) was recorded daily. Blastocysts were graded according to the degree of expansion and quality of the inner cell mass and trophectoderm. Implantation rates were considered per embryo transfer. Miscarriage was defined as a pregnancy failing to reach the 24th week of gestation after detection of a gestational sac(s).

#### 4. Statistical analysis

We performed a study of a cohort of 578 women who underwent ART treatment. Initially we conducted an exploratory data analysis using graphical techniques and quantitative analysis in order to characterize the sample, and detect possible extreme outliers and measurement errors. Data were stratified into two groups, according to the women's metabolic risk, as based on their WC <sup>[20]</sup>: lower metabolic risk (WC < 88 cm) and higher metabolic risk (WC ≥ 88 cm).

To investigate the existence of differences between women with a WC  $\geq$  88 cm and those with a WC < 88 cm, we conducted a one-way ANCOVA (analysis of covariance), assuming equal variances (Levene's test for equality of variances). To study the association between WC and ART treatment outcomes, we calculated Fisher's exact test and risk estimate (*odds ratio*). The assumptions of the statistical techniques used were validated.

Post-hoc power calculations demonstrated that the sample size achieved was sufficient to detect medium effects [f=.15, p<.05, power = .95, G\*Power 3] in the ANCOVA <sup>[27]</sup>.

<b>Table 1a</b> clinical characteristics of the conort of datients (5/8 wo
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Statistical analysis was performed with the support of IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp, with the level of significance fixed at 5%.

#### Results

A total of 610 couples was enrolled in the study. Of these, 24 were lost to follow-up and a further 8 couples were excluded from the study because they failed to initiate treatment due to personal reasons (n=6) or spontaneous pregnancy (n=2). The final sample consisted of 578 couples.

The women's mean age (years) was  $35 \pm 3$  and almost all were Caucasian (99.5%). Primary infertility was present in 77% of the couples. A short antagonist protocol was used in 51.2% of cycles, a long agonist protocol in 37%, and a short agonist protocol in 6.6%. FSHr was used in 62.8% of cycles and hMG in 37.2%.

Most of the women were of normal weight (63%), 3.1% were underweight, 24% were overweight, and 9.9% were obese. According to their WC measurement, 82.5% of women had a lower metabolic risk (WC < 88 cm) and 17.5% a higher metabolic risk (WC  $\geq$  88 cm). The clinical characteristics of the cohort of patients are presented in Table I (A, B). It is interesting to note that not all the obese women had a higher metabolic risk (9 of these women had a WC < 88 cm). On the other hand, in the overweight and normal weight classes, there were 45 and 8 women, respectively, who had a higher metabolic risk

	MEAN (± SD) / FREQUENCY	MIN-MAX / %				
Age (years)	35 ± 3	21-40				
WC (cm)	79.4 ± 8.8	60.0-114.0				
BMI (Kg/m²)	24.0 ± 4.1	14.9-42.0				
Duration of infertility (months)	58 ± 35	12-204				
Previous IVF cycles (no.)	1 ± 1	0-5				
Smoking	88	16.1				
Main cause of female infertility						
Ovulatory dysfunction	63	16.1				
Endometriosis	65	16.6				
Tubal factor	95	24.3				
Cervical factor	7	1.8				
Unexplained infertility	121	30.9				
Male factor	40	10.2				
Weight class						
Underweight (BMI <18.5 Kg/m²)	18	3.1				
Normal weight (18.5 Kg/m <sup>2</sup> $\leq$ BMI $<$ 25.0 Kg/m <sup>2</sup> )	364	63.0				
Overweight (BMI: 25.0 Kg/m <sup>2</sup> $\leq$ BMI $<$ 30.0 Kg/m <sup>2</sup> )	139	24.0				
Obese (BMI ≥30.0 Kg/m²)	57	9.9				
WC group						
Lower metabolic risk (WC <88 cm)	477	82.5				
Higher metabolic risk (WC $\ge$ 88 cm)	101	17.5				
RMI: body mass index: WC: waist circumference: SD: standard deviation: WE: in vitro fertilization						

	WC < 88	WC ≥ 88	
	Frequency		
Underweight (BMI <18.5 Kg/m²)	18	0	
Normal weight (18.5 Kg/m <sup>2</sup> $\leq$ BMI < 25.0 Kg/m <sup>2</sup> )	356	8	
Overweight (BMI: 25.0 Kg/m <sup>2</sup> $\leq$ BMI < 30.0 Kg/m <sup>2</sup> )	94	45	
Obese (BMI ≥30.0 Kg/m²)	9	48	
BMI: body mass index: WC: waist circumference			

Table 1b Clinical characteristics of the cohort of patients (578 women).

(Table IB). The women with a smaller WC (< 88 cm) had a higher number of oocytes collected ( $\approx 8\%$  more, p=0.049), a higher number of mature oocytes ( $\approx 20\%$  more, p=0.010), a higher number of fertilized oocytes ( $\approx 22\%$  more, p=0.017), and a lower gonadotropin requirement ( $\approx 10\%$  less, p=0.042) than the women with a WC  $\geq 88$  cm. Figure 1 displays the outcomes related to oocytes retrieved and fertilization in both groups. Although the difference was not statistically significant, the women with a WC < 88 cm had a higher fertilization rate. The implantation rate was not different between groups. The comparison of ART outcomes according to WC group is presented in Table II.

There was a significant association between WC group and the occurrence of fertilization (p=0.033). The occurrence of fertilization was higher in the women with a WC < 88 cm (OR [95% CI]: 2.04 [1.04-4.00]). No significant associations were observed between WC group and pregnancy, live birth, cycle cancellation and miscarriage rates (Table III). **Figure 1** Comparison of the outcomes related to oocytes retrieved and fertilization between groups: WC < 88 cm (lower metabolic risk) and WC  $\geq$  88 cm (higher metabolic risk).



Table 2 Comparison of ART outcomes according to WC group.

	WC < 88 cm - NO.= 477	WC $\geq$ 88 cm - NO.= 101	ONE-WA	Y ANCOVA			
	Mean	± SD	F	p			
Total dose of FSH (IU) - short antagonist protocol <sup>a</sup>	1793.1 ± 659.1	1989.1 ± 681.3	4.170	0.042*			
No. of oocytes collected <sup>a</sup>	9.1 ± 5.3	8.4 ± 6.1	3.891	0.049*			
No. of mature oocytes <sup>a</sup>	$6.5 \pm 4.1$	$5.4 \pm 4.2$	6.629	0.010*			
No. of fertilized oocytes <sup>a</sup>	4.1 ± 3.0	$3.2 \pm 3.2$	5.780	0.017*			
Fertilization rate <sup>a</sup>	$63.5\pm30.8$	60.1 ± 35.0	0.315	0.238			
Implantation rate <sup>a</sup>	21.0 ± 33.4	21.1 ± 34.3	0.052	0.795			
* mean adjusted for age (years), duration of infertility (months) and no. of previous IVF cycles; * difference is significant at 0.05 (2-tailed); ART: assisted reproductive technologies; WC: waist							

circumference; BMI: body mass index; FSH: follicle-stimulating hormone; SD: standard deviation

Table 3 Association of ART treatment outcomes according to class of WC.

	WC < 88 cm - N = 477	$WC \geq 88 \ cm - N = 101$	FISHER'S EXACT TEST	RISK ESTIMATE				
Outcome	Freque	ncy (%)	p	OR (95% CI)				
Fertilization	387 (93.1)	72 (83.7)	0.033*	2.04 (1.04-4.00)				
Pregnancy								
Per cycle	97 (20.3)	17 (16.8)	0.421	1.27 (0.71-2.22)				
Per transfer	97 (32.3)	17 (32.1)	0.970	1.02 (0.54-1.89)				
Live birth (per transfer)	72 (24.0)	15 (28.3)	0.210	0.38 (0.08-1.79)				
Cancelled cycle	42 (8.8)	12 (11.9)	0.335	0.71 (0.36-1.43)				
Miscarriage	26 (26.5)	2 (11.8)	0.184	2.70 (0.58-1.25)				
* association is significant at the 0.05 level (2-tailed); ART: assisted reproductive technologies; WC: waist circumference; OR: odds ratio; CI: confidence interval								

#### Discussion

This study aimed to examine whether women with different levels of metabolic risk, as based on their WC measurement, have different ART treatment outcomes. The results showed that women with a WC  $\geq$  88 cm have poorer outcomes and that the probability of fertilization was 2 times higher in women with a lower WC.

Obese women are prone to ovulatory dysfunction as a consequence of insulin resistance <sup>[22]</sup> and altered expression of adipokines (higher levels of leptin and lower levels of adiponectin) <sup>[23,24]</sup>. The associations of obesity with insulin resistance and with hyperinsulinemia are more striking in the presence of abdominal distribution of adiposity <sup>[2]</sup>, which is in accordance with our results. Women with a WC  $\geq$  88 cm had a higher gonadotropin requirement and lower numbers of oocytes collected and of mature oocytes. Approximately half of the women with higher metabolic risk (WC  $\geq$  88 cm) were non-obese, a finding that underlines the importance of fat distribution.

Another effect of obesity is high FFA levels, which have been linked with insulin resistance (systemic levels), but also with apoptosis of human granulosa cells <sup>[28]</sup> and with poor cumulus oocyte complex morphology <sup>[29]</sup>, the latter a consequence of high levels of FFA in the follicular fluid, which correlates with plasma levels <sup>[30]</sup>. Obese women have higher levels of pro-inflammatory cytokines, insulin, lactate and triglycerides in the follicular fluid <sup>[31]</sup>. Abdominal obesity is associated with markers of oxidative stress in follicular fluid <sup>[32]</sup>, which has been associated with poor oocyte quality. The altered microenvironment of oocytes could explain our findings of impaired response to gonadotropins, as well as lower quality of oocytes in women with a WC  $\geq$  88 cm (lower number of mature and fertilized oocytes).

Despite the negative influence of abdominal obesity on ovulation and oocyte quality, the implantation and clinical pregnancy per transfer rates were not impaired in our study, suggesting that dysfunctional adipose tissue does not influence endometrial receptivity. Although there are no studies evaluating women with different WC measurements, this hypothesis is supported by a meta-analysis of IVF outcomes in obese donor oocyte recipients <sup>[33]</sup>. Moreover, a study concerning women who underwent ICSI has demonstrated that obesity has no impact on endometrial thickness, endometrial pattern and uterine blood flow. In fact, obese and overweight women who participated in the study by Zeng *et al.* had similar pregnancy and miscarriage rates to those recorded in normal weight women <sup>[34]</sup>.

Our study has an important merit. To the best of our knowledge, it is the first study concerning the influence of WC (a marker of metabolic risk) on ART treatment outcomes. The association of android fat tissue distribution (defined as WHR  $\geq 0.8$ ) with lower pregnancy rate in IVF was previously evaluated by Wass *et al.* <sup>[26]</sup>. Our data was analyzed adjusting for the women's age in order to evaluate the value of abdominal obesity in itself. We believe this is an important issue, as there is robust evidence suggesting that female age should be considered one of the stronger predictors of successful pregnancy after IVF <sup>[35]</sup>. Alongside the aforementioned strength, this study has a limitation: we did not take into account the role of male obesity and semen quality, factors that may have influenced some of our study outcomes.

Although the mechanism is not totally understood, it is widely recognized that regional abdominal fat, irrespective of the total amount of body fat, leads to metabolic complications. Therefore, the contradictory results of studies concerning the influence of obesity on ART outcomes could be explained in part by differences in fat distribution. This hypothesis has already been addressed by Wise *et al.* in spontaneous pregnancy. In their study, they found that the overweight and obese women had a lower fecundability rate (FC), ranging from 0.55 to 0.83, in comparison with the normal weight women (FC=1.00). When adjusting for women's WC, the FC was even lower, ranging from 0.48 to 0.72<sup>[6]</sup>.

In conclusion, this study highlights the importance of considering women's fat distribution when predicting ART treatment outcomes and counseling on treatments, particularly ovarian stimulation and oocyte quality.

#### **Authors' roles**

AFF designed the study, analysed and interpreted the data and drafted the article. APS collected and analysed the data. MMR analysed and interpreted the data and edited and revised the manuscript. BRC performed the statistical treatment and analysis of the data. PC and ALC collected the data and revised the article. TAS designed the study, interpreted the data and revised the manuscript. All authors read and approved the final version of the manuscript to be published.

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## Abnormal vascular architecture at the placental-maternal interface in preeclampsia

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#### ABSTRACT

**Background and purpose:** The aim of this study was to characterize the vascular architecture in the placental bed in pregnancies complicated by preeclampsia and in normal pregnancies.

**Methods:** Vessel numbers and cross-section area density in 11 preeclamptic placental beds were compared with 10 normal placental beds using computer-assisted image analysis of whole-slide CD31-immunolabeled sections.

**Results:** The total surface occupied by vessels was significantly reduced in preeclamptic placental beds compared with controls beds. However, the number of vessels/section and average surface were similar in all cases. Vessel size distribution differed between the two groups: more smaller vessels were found in preeclamptic placental beds.

**Conclusions:** Using a whole slide scanning and computer-assisted analysis method, we demonstrated a different morphological architecture of vessels in the placental beds of preeclamptic patients which might reflect the previously reported findings of insufficient trophoblast invasion and incomplete vascular remodeling.

#### **KEYWORDS**

Preeclampsia; placental bed, vessel architecture, virtual imaging.

#### Introduction

Preeclampsia (PE) is a pregnancy-associated disorder and a multisystem disease characterized by the sudden onset of hypertension associated with either proteinuria or end-organ dysfunction after 20 weeks of gestation in women with no previous history of hypertension. PE is one of the most important causes of maternal and perinatal morbidity and mortality, with PE-related deaths estimated to amount to 50,00-60,000 per year. The incidence of PE has increased by 25% in the United States over the past two decades <sup>[1]</sup>.

The clinical manifestations are caused by mild to severe microangiopathy of different organs<sup>[2]</sup>. Hepatic or renal failure, pulmonary edema, cardiovascular disease and death are the potential maternal sequelae. On the fetal side, potential consequences of PE are late miscarriage, retro-placental hematoma, fetal growth restriction (FGR), hypoxic neurologic injury and in utero fetal death. These fetal and neonatal consequences result from placental hypoperfusion.

Several genetic, immunologic, physiological, environmental, demographic and pregnancy-associated factors have been associated with the occurrence of PE. These include: multiple pregnancy, maternal age at the extremes of the fertile range, personal and family history of PE, obesity, ethnic group, egg donation, chronic hypertension, renal disease, metabolic disease and thrombophilia <sup>[3,4]</sup>.

Although the etiology of PE has not been completely elucidated, the placenta plays a central role in the development of this disease. The prevailing view, supported by epidemiologic and experimental data, is that PE is due largely to a defect in

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trophoblast invasion and the remodeling of the uterine spiral arteries <sup>[5]</sup>. It results in impaired placentation in the first trimester of pregnancy, leading to inadequate placental perfusion in the second and third trimesters <sup>[6]</sup>. This process leads to an imbalance between pro-angiogenic and anti-angiogenic factors, released by the placenta into the maternal circulation, that causes systemic endothelial dysfunction <sup>[7]</sup>. However, a precise definition of the vascular architecture at the placental-maternal interface is lacking in women with PE.

The aim of this study was to characterize the vascular architecture in the placental bed in pregnancies complicated by PE and in normal pregnancies, applying our previously published method consisting of high-resolution virtual imaging of whole-tissue sections and computer-assisted image analysis of the placental bed of PE patients and controls<sup>[8]</sup>.

#### **Methods**

#### **Tissue collection**

The American College of Obstetricians and Gynecologists (ACOG) defines PE as the onset, after 20 weeks of amenorrhea,

of hypertension ( $\geq$  140/90) associated with proteinuria and/or single or multiple organ involvement <sup>[9]</sup>. Placental bed biopsies were obtained prospectively from 11 patients presenting PE and from 10 women presenting uncomplicated pregnancies and delivered by elective cesarean section for breech presentation, feto-pelvic disproportion or a history of two previous cesarean sections. All these biopsies were sampled under direct vision by the same operator (F.C.); samples were obtained after placental delivery from the central zone of the placental site applying the previously described knife technique <sup>[10,11]</sup>. Adequacy of the samples was controlled by analysis of hematoxylin-eosin– stained sections. Only areas in which myometrium was present were analyzed. These placental bed biopsies were analyzed by two operators (F.C. and J.T.).

#### Histology and immunohistochemistry

All biopsies were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. Immunohistochemistry for CD31 (vascular endothelial cell marker) was performed on 4-µm thick paraffin sections that were mounted on aminopropyltriethoxysilane (Tespa)-coated glass slides.

The sections were dewaxed in xylene and rehydrated. Antigen retrieval was achieved using Target Retrieval Solution (S2031; DakoCytomation, Glostrup, Denmark). Endogenous peroxidases were blocked by incubation in 3% H2O2 for 20 minutes, and nonspecific binding was prevented by incubation in PBS/normal goat serum. Sections were incubated with monoclonal anti-CD31 (M0823, 1:40; DakoCytomation) for 60 minutes at 37°C and then biotinylated goat antimouse IgG (E0433, 1:400; DakoCytomation), followed by streptavidin/ horseradish peroxidase (P0397, 1:500; DakoCytomation).

Labeling was visualized with 3-3-diaminobenzidine hydrochloride (K3468; DakoCytomation) as the chromogen and hematoxylin as the counterstain. Nonspecific binding of primary antibody was evaluated using mouse IgG1 at the same immunoglobulin concentration as the primary antibody (X0931; DakoCytomation).

#### Image acquisition, processing, and measurements

Virtual images of the whole tissue sections were acquired using the fully automated digital microscopy system dot-Slide (BX51TF; Olympus, Aartselaar, Belgium) coupled with a Peltier-cooled, high-resolution digital color camera (XC10; Olympus) at a resolution of 1376 x 1032 pixels. Whole-tissue sections at high magnification (x100) were scanned, yielding virtual images with a 0.65  $\mu$ m pixel size.

#### Table 1

The vessel parameters were characterized in the myometrium located under the placental-myometrial junction. Binary images were decimated before quantification, according to a previously described procedure because the original virtual images exceeded several gigabytes. CD31-identified endothelial cells allowed automatic computerized acquisition of blood vessel characteristics. Vessel area density was defined as the number of pixels belonging to all vessels (endothelium and encompassed lumen, i.e., the total area occupied by vessels) divided by the total number of pixels of the myometrial area. The number of vessel cross-sections per unit area was defined as the number of vessels divided by the area of myometrium. The area of each vessel cross-section was measured, and the vessel cross-section area distribution was plotted as a histogram. Image processing and measurements were performed using MAT-LAB version 9.2 software (MathWorks, Natick, MA).

#### **Statistical analysis**

The results are expressed as means ± 95% CI of individually tested parameters (vessel area density, number of vessels/unit area, area of individual vessel sections, vessel cross-section area distribution, and distance from each vessel to the placental-myometrial junction) (Table 1). For each parameter, a large number of measurements was performed in each individual (mean number of vessels per tissue section,  $3385 \pm 850.8$  for PE cases and  $2863 \pm 466.5$  for control cases). Tests of homogeneity performed in each group (PE and control) confirmed the homogeneity of both populations for the evaluated parameters. It can therefore be concluded that for the tested parameters, the PE and the control group displayed homogenous characteristics. The statistical differences between the groups were assessed using the nonparametric Mann-Whitney U test. Furthermore, we used the nonparametric Kolmogorov-Smirnov test for distributions and analysis of covariance for linear regressions. P < 0.05 defined significance. Statistical analyses were performed with MATLAB version 9.2 software.

#### Results

Table 2 summarizes the demographic and obstetric characteristics of the PE patients and controls. There was no significant difference in gravidity and parity between the two groups. As expected, women with PE had higher blood pressure and abnormal proteinuria. Furthermore, the women with PE delivered significantly earlier than the controls.

	VESSEL SURFACE DENSITY		NUMBER O	F SECTIONS	AVERAGE SURFACE		
	СТ	PE	CT	PE	CT	PE	
Mean	0,09694	0,06079	80,13	64,9	0,001453	0,000962	
SD	0,05303	0,01903	50,54	16,03	0,000662	0,000309	
SEM	0,01677	0,005738	15,98	4,833	0,000209	9,31E-05	
Lower 95% CI of mean	0,05901	0,04801	43,98	54,14	0,00098	0,000754	
Upper 95% CI of mean	0,1349	0,07358	116,3	75,67	0,001927	0,001169	

#### Table 1 Demographic and obstetrical characteristics.

VARIABLE	CONTROLS (N=10)	CONTROLS (N=10) PREECLAMPSIA (N=11)					
Maternal age at delivery (years) <sup>a</sup>	29 (20- 42)	28 (23- 38)	NS				
Gestational age at delivery (weeks) <sup>a</sup>	38.5 (32.7-39)	32 (25.3- 37.1)	0,00003				
Gravidityª	3 (1-8)	3 (1-6)	NS				
Parity <sup>a</sup>	2 (0-7)	1 (0-2)	NS				
HELLP	0/10	3/11					
Proteinuria	0/10	10/11					
Systolic BP (mmHg)	121 (101-155)	145 (125-185)	0,021				
Diastolic BP (mmHg)	72.8 (62-105)	88.18 (71-105)	0,032				
Birth weight (g)	3240 (1660-3838)	1540 (480- 2258)	0,0004				
NS not significant <sup>a</sup> Data presented are expressed in medians (minimum-maximum) and analyzed with the Mann-Whitney II test							

Figure 1 illustrates the computer-assisted image algorithm for a control and a PE case. On the whole-slide section most vessels were automatically detected, whereas some missed vessels and the limits of tissue section were manually corrected. Figure 1 (B and E) shows binarized images, subsequently used for vascular architecture characterization.

#### **Placental bed vessel characteristics**

The mean number of vessels per tissue section was similar in the control and PE groups (80.13 ±0.13 vessels/mm<sup>2</sup> vs 64.90  $\pm$  4.83 vessels/mm<sup>2</sup>, P= 0.8053, figure 2A). However, in the PE group the cross-sectional vessel areas were reduced compared with what was observed in the control group (0.0608  $\pm$ 0.0057 vs  $0.0969 \pm 0.0168$ , P = 0.0378, figure 2B). The surface occupied by blood vessels was determined and found to be slightly reduced in PE (0.0962  $10^{-3} \pm 0.0932 10^{-3}$  vessels/mm<sup>2</sup> vs  $1.453 \ 10^{-3} \pm 0.294 \ 10^{-3}$  vessels/mm<sup>2</sup>, *P*=0.0620, figure 2C). To confirm this, the log<sub>10</sub>-transformed vessel size distribution was analyzed (Figure 2D). This normalized histogram indeed highlights the presence of smaller vessels in PE.

#### Discussion

In this study, we compare the vessel organization in the placental bed of PE cases compared with the placental bed in normal pregnancy applying a method previously used by our workgroup to describe vascular characteristics in early invasive cervical cancer and placenta increta [8,12]. This method combines the use of a high-resolution virtual imaging system on whole-tissue sections with the use of computer-assisted image analysis of whole-slide CD31-immunolabeled sections. Through this method, we demonstrated and confirmed that more smaller vessels are present in PE placental beds compared with normal placental beds.

PE is a complex disorder with a large range of clinical pres-

Figure 1 Illustration of the computer-assisted image analysis of vessels in control sections (A-C) and PE sections (D-F). A and D, representative CD31-positive immunolabeled vessels. B and E, binarized images generated from from immunolabeled sections. C and F, selected zones at higher magnification. Figure 1G shows a close up of the CD31 staining.





**Figure 2** A, Mean number of vessels per cross-section. B, Vessel area density. C, Mean vessel cross-section area. D, Histogram of the normalized frequency of vessels versus vessel cross-section area distribution (log<sub>10</sub> scale).

entations. This heterogeneity suggests the possible existence of various pathogenic mechanisms. It is well known that PE is associated with poor placentation and incomplete remodeling of the utero-placental spiral arteries<sup>[13]</sup>.

Despite extensive study of the pathogenesis of PE, the exact vascular architecture of the placental bed in PE is unclear. As regards the placenta, increased villous capillary branching has been reported in placentas from PE versus normal term pregnancies <sup>[14]</sup>. In contrast, other investigators have reported decreased micro-vessel counts in PE placentas <sup>[5, 15, 16]</sup> or unchanged vascular densities between PE and normal term placentas <sup>[17, 18]</sup>. As early as 1985, Las Heras *et al.* showed a significant reduction in the *ratio* of lumen-to-whole-diameter of the fetal arteries in "toxemia" as compared with normal pregnancy and acute fetal distress groups <sup>[19]</sup>. Moreover, they showed that the mean lumen-to-whole-diameter *ratio* also differed between regions of the placenta in all groups, the most marked reduction being in the parachorial region and the least prominent reduction in the parabasal zone. Finally, they found no significant differences in the mean diameter *ratio* between three subgroups of the toxemic pregnancies (PE, essential hypertension and renal disease groups).

More recently, Uras *et al.* evaluated placentas of PE cases by using immuno-histochemical staining with CD31 and with Factor VIII antibodies. When the PE and control groups were compared, CD31 and Factor VIII staining were significantly lower in the PE group. This study indirectly suggests that the balance between proangiogenic and antiangiogenic factors is shifted in favor of anti-angiogenic factors in PE <sup>[15]</sup>.

In 2013, Lyall *et al.* demonstrated the occurrence of a major defect in myometrial spiral artery remodeling in PE<sup>[5]</sup>. In this study, placental bed biopsies were immuno-stained to determine vessel wall integrity, extravillous cytotrophoblast location/density, periarterial fibrinoid, and endothelium. The authors compared normal pregnancies and pregnancies complicated by PE or severe FGR and they examined spiral artery

remodeling and extravillous cytotrophoblast density. The invasion of vessel wall smooth muscle was reduced in myometrial spiral arteries in PE and FGR groups compared with controls. Although failure to destroy myometrial vessel wall smooth muscle is a feature of PE, 10% of decidual vessels in PE also retained their muscle wall. Moreover, interstitial extravillous cytotrophoblast density seems to be similar in normal pregnancy and PE and the normal group showed more intramural extravillous cytotrophoblasts than the PE group. This work suggests that lack of intramural trophoblasts in the myometrial vessels rather than defective interstitial trophoblast invasion may be the primary abnormality in PE<sup>[20]</sup>. In our study, we focused on the size and the areas occupied by vessels. We did not perform detailed analysis of the histologic appearance of the different vessels in the placenta beds.

Possible limitations of this study are the small sample size and the two-observer analysis. Moreover, another possible limitation is the difficulty in collecting gestational age matched bed biopsies from normal pregnancies as controls. However, the median 5-week difference in gestational length between our groups should not drastically affect vessel size and distribution in the placental bed. We chose patients undergoing elective term cesarean section deliveries as the control group in order to obtain placental bed biopsies of good quality and to avoid the bias of impaired placentation in earlier deliveries often indicated for pregnancies complicated by PE, intrauterine growth restriction (IUGR) or premature rupture of membranes.

This study confirms previously published data on placental bed abnormality in pregnancies complicated by PE. In this clinical situation, vessels are smaller in size, which can explain the under-perfusion of the placenta. Compared with manual or stereotaxic methods, the automated detection of CD31-stained histologic sections used in this study allows easier and probably more precise measurement of the number and size of vessels.

#### Conclusion

Through whole slide scanning and computer-assisted analysis, we revealed a different morphological architecture of vessels in the placental bed in PE. This might reflect the insufficient trophoblast invasion and incomplete vascular remodeling previously described by others.

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#### Retrospective observational study: the natural history of cervical intraepithelial neoplasia during pregnancy

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#### ABSTRACT

**Aims:** To assess the persistence, regression and progression of cervical dysplasia in pregnancy, within a tertiary London hospital.

**Methods:** All cases referred for colposcopy at Kings College Hospital during pregnancy, between September 2011 and August 2017, were retrospectively identified. Women underwent a tissue biopsy only if clinically indicated. All women were seen for colposcopy between 3 and 6 months postnatally. Women were excluded from the final analysis if they were referred for reasons other than an abnormal smear, and if follow-up data were not available.

**Results:** 82 pregnant patients (median age: 35 years, range: 27-48 years) were seen during this period. 56 cases were referred for colposcopy due to abnormal smears and had complete follow-up data; the other cases were excluded. 24 had high-grade cervical intraepithelial neoplasia (CIN). Of these, CIN regressed in 6/24 (25%) cases and persisted, necessitating excisional treatment, in 18/24 cases (75%). No cervical cancer cases were diagnosed. The regression, persistence and progression rates of the remaining 32 cases with low-grade smear abnormalities during pregnancy were: 20/32 (63%), 6/32 (19%) and 6/32 (19%) respectively.

**Conclusion:** This study shows high rates of regression of low-grade abnormalities following pregnancy. Additionally, there were no cases of progression of high-grade CIN to cancer, thus supporting safe conservative management of these women. Post-partum follow-up remains essential for those with high-grade CIN due to significant levels of persistence.

#### **KEYWORDS**

Colposcopy, CIN, pregnancy, pregnant.

#### Introduction

Cervical cancer is the most common gynaecological malignancy diagnosed during pregnancy. Its incidence is estimated to be 1.2-4.5 per 10,000 women <sup>[1-3]</sup>. Most pregnancies occur between the ages of 18 and 35 years, the same range that sees the peak incidence of cervical intraepithelial neoplasia (CIN). Accordingly, the prevalence of CIN in the pregnant population is approximately 1% <sup>[4]</sup>.

Several studies have looked at the evolution of CIN in pregnancy. The risk of progression of low-grade CIN is thought to be small (6-14%)<sup>[5,6]</sup> with high rates of regression to normal after pregnancy, compared with matched non-pregnant controls <sup>[6]</sup>. This is in concordance with British Society of Colposcopy and Cervical Pathology (BSCCP) guidelines, and the American Society for Colposcopy and Cervical Pathology Consensus guidelines (ASCCP), which recommend colposcopic examination in pregnancy, if CIN 1 or less is suspected, and to repeat colposcopic examination 3 months postnatally <sup>[7,8]</sup>.

Studies examining the regression, persistence and progression rates of high-grade CIN are much more variable. Serati *et al.*, in one of the most cited studies, prospectively followed up 78 women who underwent a PAP smear at between 8 and 17

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weeks' gestation and had abnormal cytology <sup>[6]</sup>. In their cohort of patients, at the first postpartum follow-up appointment, 53% of the women who had shown high-grade disease had persistent high-grade CIN and subsequently underwent large loop excision of the transformation zone (LLETZ), 27% showed complete regression, and 20% regression to CIN 1.

Fader *et al.* retrospectively analysed 1079 pregnant patients referred for colposcopy, of whom 164 had high-grade cytology <sup>[9]</sup>. Of these, only 36 patients had either biopsy-confirmed CIN 3 or colposcopic impressions of CIN 3. The regression rate was lower than Serati *et al* study, at 32%, with persistence rate of 68%. Due to the retrospective nature of this study, the follow-up rate was low, at approximately 50%. Other studies have reported regression rates of between 17 and 70% for high-



#### grade CIN [10,11].

The aim of this study was to assess the persistence, regression and progression of cervical dysplasia in pregnancy, within a tertiary London hospital.

#### Methods

All women referred for colposcopy at Kings College Hospital during pregnancy, between September 2011 and August 2017, were retrospectively identified.

Data collected from ViewPoint software used for colposcopy included: demographic details, such as age and smoking history. In addition, gestational age at time of colposcopy, referral cytology, colposcopic impression and results of biopsy (if performed) were recorded. All patients underwent colposcopic examination performed by one of two highly experienced colposcopists. Women only had a tissue biopsy if clinically indicated, for example, those with suspicious lesions on colposcopy, or if there was a significant discrepancy between colposcopic and cytological findings.

The inclusion criteria were all women referred for colposcopy during pregnancy with an abnormal smear and seen both during pregnancy and between 3 and 6 months postnatally. The colposcopy findings had to be documented.

Women were excluded from final analysis if they were referred for reasons other than an abnormal smear, and if follow-up data were not available.

#### Results

82 pregnant patients were seen during this period. Their median age was 35 years (range 27-48 years). Seventy-three percent were parous and 10% were smokers. Twenty-three percent of the women had previously undergone LLETZ treatment. The women were seen at the colposcopy clinic antenatally at between 3 and 36 weeks' gestation.

Twenty-nine (35%) patients were referred due to highgrade smear abnormalities, and 38 due to low-grade abnormalities (46%). The remaining women were referred for reasons other than an abnormal smear, including: follow up of glandular disease (2%), vaginal bleeding (9%) and an abnormal-looking cervix (6%) – all these were excluded from further analysis.

Patients were also excluded if they had inadequate follow-up data (15 cases).

A total of 56 cases remained; of these 24 had high-grade CIN and 32 low-grade CIN. Only one patient underwent a punch biopsy during pregnancy for an abnormal-looking cervix. No complications associated with the punch biopsy were recorded.

Among the cases with high-grade abnormality in pregnancy, CIN regressed in 6/24 (25%) cases and persisted, necessitating excisional treatment postnatally, in 18/24 cases (75%). The 6-month test of cure smear was normal in all those who underwent treatment. No cervical cancer cases were diagnosed.

Among the women who showed low-grade smear abnormalities during pregnancy, the regression, persistence and progression rates were 20/32 (63%), 6/32 (19%) and 6/32 (19%) respectively. All those who progressed had LLETZ treatment postnatally, which confirmed high-grade CIN on histology.

Pregnancy outcomes were available only for 19/56 cases. Of these, 14/19 (74%) had vaginal deliveries, 4/19 had Caesarean sections (21%) and 1/19 had a miscarriage (5%).

#### Discussion

This study supports the BSCCP guidelines on conservative follow up of pregnant women with low-grade smear abnormalities postnatally, given the high rate of regression observed (63%). This finding is also consistent with other studies <sup>[5,6,12]</sup> that showed high regression rates of CIN 1, particularly when compared with a non-pregnant cohort <sup>[6]</sup>.

It is well known that the cervix undergoes many physiological changes during pregnancy, which can make colposcopic examination challenging. It has been suggested that some of the physiological changes, such as those due to the interaction between oestrogen and HPV, may play a role in the increased regression rates seen postnatally <sup>[6]</sup>. There has also been some debate as to whether mode of delivery is associated with higher rates of regression <sup>[13-15]</sup>. Inadequate pregnancy outcome data in the present study limits any analysis focusing on mode of delivery.

Women with a history of treatment of cervical dyskaryosis are at higher risk of future abnormalities later in life <sup>[16]</sup>. In keeping with this, in this study, a significant proportion (23) of women with abnormal smears in pregnancy had already had at least one LLETZ treatment in the past.

Reassuringly, there were no cases of micro-invasive or invasive cancer in this cohort. This supports expectant management of high-grade CIN, in pregnancy too, and is in concordance with other studies which found very low rates of evolution to cancer <sup>[11]</sup>. There was, however, a very high rate of persistence of high-grade CIN (75%), which underlines the need for close follow up of these women postnatally. The variations in high-grade CIN regression and persistence rates observed in different studies (0-70% and 38-100%, respectively) may be due to differences in follow-up durations and populations and to variations in the methods used to confirm regression and persistence <sup>[5,11,17]</sup>.

Many studies have shown that cervical biopsy is safe in pregnancy <sup>[18]</sup>. Indeed, because cytology and colposcopic examination alone can be inadequate for the evaluation of cervical abnormalities during pregnancy <sup>[18-20]</sup>, patients with suspicious lesions should undergo a biopsy if there is doubt. There is significant variation amongst colposcopists regarding thresholds to perform cervical biopsies in pregnancy. In the present study only one patient had a cervical biopsy in pregnancy, with no complications. Many additional studies have shown that excisional treatment with LLETZ is also safe in the first trimester <sup>[21,22]</sup>. Siegler *et al.* performed LLETZ (also known as the loop electrosurgical excision procedure) in 43 pregnant patients with high grade CIN during the first 15 weeks of gestation <sup>[22]</sup>. There were no cases of major haemorrhage. Additionally, 92% had term deliveries.

One of the difficulties in evaluating studies of the evolution of CIN in pregnancy lies in the heterogeneous nature of diagnosis of high-grade CIN. In the present study, the diagnosis was based on smear and colposcopic examination, as biopsy was only performed if clinically indicated. This is different to several studies in which biopsy confirmation of CIN was among the inclusion criteria <sup>[6,11,17,23]</sup>. In the absence of histology there is a risk of misdiagnosis, given the difficulty of obtaining accurate colposcopic assessment in pregnancy [18,19]. In the present study, this difficulty was mitigated by the fact that all assessments were performed by one of two experienced colposcopists.

A limitation of this study is the size of the cohort, even though it is comparable with those of other studies looking at the evolution of CIN in pregnancy. In addition, due to its retrospective nature, 18% of the original cohort were lost to follow up, which could introduce potential bias.

In conclusion, this study showed a high rate of regression of low-grade abnormalities following pregnancy. Additionally, there were no cases of progression of high-grade CIN to cancer, thus supporting safe conservative management of these women. Post-partum follow-up remains essential for all pregnant women with dyskaryosis, although this applies particularly to those with high-grade CIN due to significant levels of persistence.

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# The impact of total body fat mass, and of its distribution in the trunk, on thyroid hormone levels after complete weight restoration, with or without recovery of menses, in adolescents with anorexia nervosa

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#### ABSTRACT

**Background and purpose:** To determine the impact of total body fat mass, and of its distribution in the trunk, on thyroid hormone levels after complete weight restoration, with or without recovery of menses, in adolescents with anorexia nervosa (AN).

**Methods:** Prospective study of 60 adolescents with AN and amenorrhea. Anthropometrics, body composition and hormonal studies were obtained at the beginning of the study and at complete weight restoration, whether (Group A) or not (Group B) menses were recovered.

**Results:** In both groups, free triiodothyronine (FT3), free thyroxine (FT4) and thyroid-stimulating hormone (TSH) levels were statistically significantly higher (p<0.001) at the end of the study compared with the time of first attendance. At weight restoration, a statistically significantly positive correlation was found between total body fat mass (Kg and %), trunk fat mass (Kg and %) and FT3 and FT4 levels in both Group A and Group B adolescents. At the same time point, trunk/extremities fat *ratio* was found to be statistically significantly positively correlated with FT3 (r=0.586, p<0.001) and FT4 (r=0.512, p<0.01), but not with TSH, in girls who recovered their menses, in contrast with adolescents who remained amenorrheic.

**Conclusions:** Total body fat mass and its distribution in the trunk were found to be statistically significantly positively correlated with FT3 and FT4 in adolescents with AN who completed restored their weight and recovered their menses.

#### **KEYWORDS**

Anorexia nervosa, body composition, thyroid dysfunction, adolescence, amenorrhea.

#### Introduction

Anorexia nervosa (AN) is a disease primarily affecting girls: 95% of cases are female adolescents <sup>[1]</sup>. Its prevalence ranges from 0.5% to 1%, although some studies report higher levels <sup>[2,3]</sup>. The new DSM-V criteria for the diagnosis of AN were recently published. The main difference in these new criteria is the deletion of criterion D, requiring amenorrhea for diagnosis of the disease <sup>[4]</sup>.

It is well known that starvation affects the hypothalamic-pituitary-thyroid axis, leading to decreased plasma free triiodothyronine (FT3) concentration, along with decreased plasma free thyroxine (FT4) and increased plasma reverse triiodothyronine (rT3) levels; thyroid-stimulating hormone (TSH) concentration is usually normal, although decreased levels of this hormone have been reported <sup>[5,6]</sup>. This picture represents what is known as "euthyroid sick syndrome" <sup>[7,8]</sup>. The low levels of circulating T3 have the effect of reducing energy expenditure and muscle protein catabolism. Weight gain leads to elevation of FT3 and decrease of rT3 levels.

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Interestingly, AN adolescents present many clinical features of hypothyroidism (bradycardia, hypothermia, delayed ankle reflexes), which lead to energy conservation. However, thyroid hormone treatment is inappropriate, leading to further weight and muscle mass loss <sup>[9]</sup>. Notably, resting energy expenditure (REE) has also been found to be significantly lower in adolescents with AN compared with healthy controls, seemingly representing an adaptive mechanism to chronic starvation <sup>[10,11]</sup>. Furthermore, the finding that re-feeding and weight restoration in girls with AN leads to increase of REE, related to changes occurring in T3 levels, highlights the role of T3 in the regulation of energy hemostasis <sup>[12]</sup>. Finally, starvation in AN in combination with lowered metabolic rate leads to increased plasma cortisol levels (as starvation stimulates gluconeogenesis and decreases peripheral glucose utilization), as well as decreased gonadotropin levels, which are also expressions of adaptive mechanisms.

#### **Materials and Methods**

A hundred and ninety-one female adolescents presented at our Division <sup>[13]</sup> with secondary amenorrhea and a body mass index (BMI)  $<5^{th}$  percentile for age (based on BMI charts for Greek females). Of these, 94 fulfilled the DSM-IV diagnostic criteria for AN and 60 were finally included in our prospective study. All girls were diagnosed with restricting-type AN; 34 adolescents were excluded from the study for the following reasons: a past history of hypothyroidism (n=10), smoking (n=12), and use of hormone replacement therapy (n=12).

At first attendance, a thorough medical history was recorded, including any past medical history, family history, medication or surgeries. Demographics were also taken into consideration. All girls were provided with menstrual calendar in which age at menarche, minimum and maximum cycle length, menstrual irregularities and time of secondary amenorrhea were recorded. Finally, the adolescents also provided information on past use of hormonal medication, age at diagnosis of AN, BMI at diagnosis of AN, physical activity (measured in hours/ week), nutritional habits and life stress events. All the girls included in the study were non-smokers, with no medical history of any other endocrine disorder, while hormonal contraceptive use had been stopped for a minimum of 6 months prior to the time of first attendance. Informed consent to participate in the study was obtained from the adolescents and/or their parents, if needed, while the protocol for the research project, which conforms with the provisions of the Declaration of Helsinki, was approved by the ethics committee of our institution.

All adolescents underwent routine pedogynecological examination, with assessment by Tanner stages of breast and pubic hair development, while BMI (calculated by dividing weight in kilograms by the square of height in meters), waist circumference, hip circumference and waist circumference/ hip circumference ratio were calculated. Additionally, blood samples were obtained between 8:00 and 10:00 AM after overnight fasting, always at the same time in the morning for the determination of endocrine profile. After coagulation, the samples were centrifuged and the serum was stored at -20°C until further processing. 17b-estradiol, prolactin, insulin, FT3, FT4 and TSH were measured by chemiluminescent immune assays, while follicle-stimulating hormone, luteinizing hormone and leptin were measured by immunoradiometric assay and cortisol was analyzed by radioimmunoassay. Pelvic ultrasonography was performed in all adolescents to exclude any pathology of the uterus and/or the ovaries, while full body composition analyses were performed based on (dual energy X-ray absorptiometry) DXA scans (GE Lunar Prodigy apparatus enCore, 2008, USA); these analyses included measurement of total body fat mass (Kg and % of total body tissue), trunk fat mass (Kg and %

of total trunk tissue) and free fat mass (Kg and % of total body tissue). Finally, trunk/extremity (right arm and left arm + right leg and left leg) fat *ratio* was calculated.

The abovementioned examinations were performed in all adolescents at first attendance. Subsequently, the girls followed a nutritional rehabilitation program under the supervision of a nutritionist until complete weight restoration. This was set at BMI>10th<85th percentile for age, with an increase in weight of over 85% of the initial weight loss, which resulted to amenorrhea. In 35 adolescents recovery of menses (two consecutive menstrual cycles) was observed after weight gain (Group A), while 25 girls remained amenorrheic for at least 6 months after complete weight restoration (Group B). The Group A and Group B girls underwent the same examinations at first attendance, and at the end of follow up. Statistical analysis was performed using RStudio, while a t-test was used for comparisons both before and after complete weight restoration. Pearson's correlation coefficient was used in order to examine the correlation between variables and how they affect each other. The level of statistical significance was set at p<0.05, while the cutoff point in Pearson's correlation coefficient was set at 0.3.

#### **Results**

Table 1 summarizes all the characteristics examined in Group A at first attendance and after menstrual recovery, as well as the same characteristics examined in Group B at first attendance and after complete weight restoration. All data are presented as mean values, with ranges and standard deviation. The mean TSH, FT3 and FT4 levels recorded in Group A were  $3.21 \pm 0.48$  mIU/l,  $1.85 \pm 0.55$  pg/ml and  $0.67 \pm 0.24$  ng/dl respectively at first attendance, and  $3.81 \pm 0.45$  mIU/l,  $3.82 \pm 0.49$  pg/ml and  $1.27 \pm 0.75$  ng/dl respectively at menstrual recovery. In Group B, the mean TSH, FT3 and FT4 levels were  $3.08 \pm 0.5$  mIU/l,  $1.61 \pm 0.69$  pg/ml and  $0.59 \pm 0.16$  ng/dl respectively at first attendance, and  $3.4 \pm 0.55$  mIU/l,  $2.94 \pm 0.71$  pg/ml and  $1.08 \pm 0.65$  ng/dl respectively at the time of complete weight restoration.

Comparing Group A and Group B at first attendance, no statistically significant difference was found in TSH (p=0.3), FT3 (p=0.15) and FT4 (p=0.21) values; instead on comparing the two groups at the time of complete weight restoration a statistically significant difference was found in TSH (p<0.001), FT3 (p<0.001) and FT4 (p<0.01). Evaluating Group A at first attendance and at the time of menstrual recovery, statistically significant differences were found in TSH (p<0.001), FT3 (p<0.001) and FT4 (p<0.001) levels between the two time points; similarly, statistically significant differences in TSH, FT3 and FT4 (p<0.001) levels were found in Group B when comparing the values at first attendance and after complete weight restoration. Table 2 summarizes all the comparisons between variables of Group A and Group B at first attendance and after complete weight restoration.

To explore the correlations between the variables contemplated in this study, we used Pearson's correlation coefficient. At the time of menstrual recovery, in Group A, total body fat mass (%) was found to be significantly positively correlated with FT3 Table 1 Group A and Group B characteristics at time of first attendance and after menstrual recovery and complete weight restoration respectively. All data are presented as mean values, with ranges and standard deviation.

	GROUP A AT FIRST GROUP A AT MENSTRU ATTENDANCE RECOVERY		MENSTRUAL Very	GROUP B AF Resto	TER WEIGHT Ration	GROUP B AT FIRST ATTENDANCE		
	Mean ± SD	Ranges	Mean ± SD	Ranges	Mean ± SD	Ranges	Mean ± SD	Ranges
Age at menarche (years)	12.61 ± 0.34	12-13.5	-	-	12.56 ± 0.32	12-13.11	-	-
Age at first attendance (years)	16.83 ± 0.75	15-18	-	-	17.03 ± 0.79	15.7-19	-	-
Time from last menstrual period (months)	16.74 ± 2.46	11-22	-	-	21.24 ± 2.74	17-27	-	-
Total weight loss before first attendance (Kg)	8 ± 1.09	6-11	-	-	10.32 ± 1.77	7-14	-	-
Total weight gain (Kg)	-	-	7.51 ± 1.52	5-13	-	-	8.24 ± 1.92	5-12
Time needed for weight gain and BMI normalization (months)	-	-	13.34 ± 2.87	8-24	-	-	13.77 ± 2.98	9-26
Time from diagnosis of AN (months)	12.57 ± 2.38	7-18	-	-	16.52 ± 2.41	12-23	-	-
Physical activity (hours/week)	7.23 ± 1.11	5-9	7.4 ± 0.88	6-9	7.57 ± 1.05	6-10	7.84 ± 1.07	6-10
BMI (Kg/m²)	16.95 ± 0.64	15.12-18.27	19.58 ± 0.62	18.6-21.4	16.75 ± 0.71	15.32-17.6	19.51 ± 1.13	15.39-21
Waist circumference (cm)	61.11 ± 1.79	57-64	65.77 ± 1.82	63-69	60.16 ± 2.72	55-65	62.92 ± 2.56	55-67
Hip circumference (cm)	78.17 ± 2.53	73-83	81.69 ± 1.92	78-86	77.48 ± 3.24	72-83	81.32 ± 3.66	72-87
WC/HC	0.78 ± 0.02	0.74-0.84	$0.8 \pm 0.02$	0.77-0.84	0.78 ± 0.01	0.75-0.8	0.77 ± 0.02	0.74-0.81
Total body fat mass (%)	17.65 ± 0.82	15.6-19.2	22.57 ± 2.39	20.1-31.4	17.26 ± 0.77	15.4-18.3	18.96 ± 0.8	16.6 – 20.2
Total body fat mass (kg)	8.62 ± 0.71	7.35-10.42	12.73 ±1.48	10.5-19.18	8.53 ± 0.8	7.39-10.06	10.84 ± 0.96	7.47-12.35
Trunk fat mass (%)	17.36 ± 0.84	14.5-18.8	22.22 ± 1.92	20-28.6	16.91 ± 1.12	12.5-18.1	18.27 ± 1.03	14.7-19.4
Trunk fat mass (kg)	3.69 ± 0.48	3.11-4.89	4.87 ± 0.73	3.87-7.34	3.55 ± 0.62	2.7-4.84	3.88 ± 0.47	2.84-5.13
Free fat mass (kg)	40.52 ± 3.15	34.74-46.77	43.91 ± 3.35	35-49.1	40.65 ± 2.62	36.85-45.43	46.28 ± 3.18	37.53-51.9
Free fat mass (%)	82.38 ± 0.79	80.8-84.4	77.51 ± 2.46	68.6-80	82.7 ± 0.83	80.9-84.6	81.08 ± 0.82	79.8-83.4
Trunk/extremities fat ratio	0.75 ± 0.02	0.71-0.82	$0.85 \pm 0.04$	0.81-0.97	0.74 ± 0.03	0.64-0.77	0.76 ± 0.03	0.67-0.79
FSH (mIU/mI)	3.97 ± 0.62	2.4-5.3	4.87 ± 0.62	3.7-6.2	3.79 ± 0.72	2.8-5.6	4.37 ± 0.68	2.9-5.9
LH (mIU/mI)	3.06 ± 0.51	2.1-4.2	4.97 ± 0.62	3.8-6.3	3.1 ± 0.57	1.2-3.9	3.48 ± 0.46	2.3-4.3
E2 (pg/ml)	16.74 ± 3.33	9-26	34.43 ± 7.52	24-52	16.28 ± 2.95	11-23	18.96 ± 2.98	12-24
PRL (ng/ml)	13.51 ± 3.06	8-19	13.69 ± 2.51	8-19	12.76 ± 1.96	10-17	12.92 ± 2.25	7-17
TSH (mIU/I)	3.21 ± 0.48	2.3-4.1	3.81 ± 0.45	2.6-4.5	3.08 ± 0.5	1.89-3.8	3.4 ± 0.55	2.21-4.2
FT3 (pg/ml)	1.85 ± 0.55	0.75-4.9	3.82 ± 0.49	2.96-5.76	1.61 ± 0.69	0.44-4.7	2.94 ± 0.73	2.1-5.1
FT4 (ng/dl)	0.67 ± 0.24	0.03-1.45	1.27 ± 0.75	0.76-2.05	0.59 ± 0.16	0.06-1.14	1.08 ± 0.65	0.57-1.75
Insulin (µU/ml)	4.41 ± 0.42	3.6-5.41	5.93 ± 0.53	4.73-6.91	4.44 ± 0.45	3.89-5.6	4.87 ± 0.54	4.06-6.1
Leptin (ng/ml)	2.88 ± 0.44	2-4.1	3.29 ± 0.52	2.4-4.6	2.76 ± 0.39	2.1-3.4	3.01 ± 0.32	2.3-3.7
BMI: body mass index, WC/HC: waist circumferen	nce/hip circumfere	ence, FSH: follicle	-stimulating horm	one, LH: luteinizin	ig hormone, E2: 17	7b-estradiol, PRL:	prolactin,	

TSH: thyroid-stimulating hormone, FT3: free triiodothyronine, FT4: free thyroxine

(r=0.566, p<0.001) and FT4 (r=0.523, p<0.01), but not with TSH (r=0.272, p>0.05); at the same time point, trunk fat mass (%) was also found to be significantly positively correlated with FT3 (r=0.477, p<0.01) and FT4 (r=0.376, p<0.05), but not with TSH (r=0.235, p>0.05). Furthermore, at the time of menstrual recovery, the trunk/extremities fat ratio was statistically significantly positively correlated with FT3 (r=0.586, p<0.001) and FT4 (r=0.512, p<0.01), but not with TSH (r=0.175, p>0.05), showing that, in the Group A girls, distribution of fat in the trunk rather than in the extremities was associated with greater improvements in FT3 and FT4 levels. Finally, at the same time point, leptin levels were found to be statistically significantly positively correlated with FT3 (r=0.609, p<0.001) and FT4 (r=0.57, p<0.01). No statistically significant correlation for the above variables in Group A was found at time of first attendance. On exploration of the correlations between these variables in Group B, after complete weight restoration, we found that total body fat mass (%) was positively correlated, to a statistically significant degree, with FT3 (r=0.309, p<0.05) and FT4 (r=0.395, p<0.05), but not with TSH (r=0.221, p>0.05), while trunk fat mass (%) was significantly positively correlated with FT3 (r=0.501, p<0.01) and FT4 (r=0.333, p<0.05), but not with TSH (r=0.199, p>0.05). In the Group B girls at the time of complete weight restoration, the trunk/extremities fat ratio was found to be positively correlated, albeit not statistically significantly, with FT3 (r=0.289, p>0.05), FT4 (r=0.201, p>0.05) and TSH (r=0.191, p>0.05). Finally, at the same time point, leptin levels were statistically significantly positively correlated

Table 2 Comparison of Group A and Group B characteristics at the time of first attendance and after menstrual recovery and complete weight restoration respectively.

	GROUP A AT FIRST Attendance	GROUP A AND B AFTER Complete Weight Restoration	GROUP A AT FIRST ATTENDANCE AND AFTER MENSTRUAL RECOVERY	GROUP B AT FIRST ATTENDANCE AND AFTER COMPLETE WEIGHT RESTORATION				
	p-value	p-value	p-value	p-value				
Age at menarche (years)	0.67	-	-	-				
Age at first attendance (years)	0.39	-	-	-				
Time from last menstrual period (months)	<0.001*	-	-	-				
Total weight loss before first attendance (Kg)	<0.001*	-	-	-				
Total weight gain (Kg)	-	0.06	-	-				
Time needed for weight gain and BMI normalization (months)	-	0.07	-	-				
Time from diagnosis of AN (months)	<0.001*	-	-	-				
Physical activity (hours/week)	0.06	0.10	0.47	0.27				
BMI (Kg/m²)	0.27	0.78	0.02*	<0.001*				
Waist circumference (cm)	0.13	<0.001*	<0.001*	<0.001*				
Hip circumference (cm)	0.38	0.65	<0.001*	<0.001*				
WC/HC	0.27	<0.001*	<0.001*	0.53				
Total body fat mass (%)	0.06	<0.001*	<0.001*	<0.001*				
Total body fat mass (kg)	0.67	<0.001*	<0.001*	<0.001*				
Trunk fat mass (%)	0.09	<0.001*	<0.001*	<0.001*				
Trunk fat mass (kg)	0.36	<0.001*	<0.001*	<0.001*				
Free fat mass (kg)	0.86	<0.001*	<0.001*	<0.001*				
Free fat mass (%)	0.14	0.01*	<0.001*	<0.001*				
Trunk/extremities fat ratio	0.07	<0.001*	<0.001*	<0.01*				
FSH (mIU/mI)	0.32	0.01*	<0.001*	<0.001*				
LH (mlU/ml)	0.78	<0.001*	<0.001*	<0.001*				
E2 (pg/ml)	0.57	<0.001*	<0.001*	0.05				
PRL (ng/ml)	0.25	0.22	0.8	0.79				
TSH (mIU/I)	0.30	<0.001*	<0.001*	<0.001*				
FT3 (pg/ml)	0.15	<0.001*	<0.001*	<0.001*				
FT4 (ng/dl)	0.21	0.01*	<0.001*	<0.001*				
Insulin (µU/ml)	0.75	<0.001*	<0.001*	<0.001*				
Leptin (ng/ml)	0.28	0.01*	<0.001*	0.15				
BMI: body mass index_WC/HC: waist circumference/bin circumference. FSH: follicle-stimulating hormone_LH: luteinizing hormone_F2: 17b-estradiol_PRI : prolactin								

TSH: thyroid-stimulating hormone, FT3: free triiodothyronine, FT4: free thyroxine. \* Statistically significant difference (p<0.05).

with FT3 (r=0.59, p<0.001) and FT4 (r=0.511, p<0.01). In this group no statistically significant correlation was found for the above-mentioned variables at the time of first attendance. Table 3 summarizes the evaluation of the variables examined with Pearson's correlation coefficient.

#### Discussion

Anorexia nervosa is known to lead to hypothalamic-pituitary-thyroid axis changes typical of the "euthyroid sick syndrome". In this case, TSH levels are usually normal <sup>[5,14]</sup>, although low TSH levels have been reported in some studies <sup>[15]</sup>, while FT3 and FT4 levels are low. The low T3 levels are a result of peripheral deiodination of T4 to rT3 rather than T3. The presence of carbohydrates appears to be important in stimulating the peripheral conversion of T4 to active T3. Interestingly, a decrease in thyroid hormone levels could contribute to the low REE observed in AN, which serves to preserve energy for vital functions <sup>[16]</sup>. Administration of exogenous thyrotropin-releasing hormone leads to blunted response of TSH in more than 50% of girls with AN <sup>[17]</sup>. As reported by many studies, weight gain and complete weight restoration leads to normalization of thyroid hormones <sup>[16,18]</sup>. This was also shown in our study. At the time of first attendance, levels of FT3 and FT4 were below normal range both in the Group A and in the Group B girls, while at the same time TSH levels were within normal range for both groups. At this time point, no statistically significant difference in FT3, FT4 and TSH levels was found between the Group A and Group B girls. After the end of the refeeding

	GR	OUP A AFTER (	COMPLETE WE	GHT RESTORA	TION AND MEN	ISTRUAL RECO	VERY		
	Total body fat mass (%)	Total body fat mass (Kg)	Trunk fat mass (%)	Trunk fat mass (Kg)	TSH (mIU/I)	FT3 (pg/ml)	FT4 (ng/dl)	Trunk / extremities fat <i>ratio</i>	Leptin
Total body fat mass (%)									
Total body fat mass (Kg)	r= 0.797*, p<0.001**								
Trunk fat mass (%)	r= 0.923*, p<0.001**	r= 0.698*, p<0.001**							
Trunk fat mass (Kg)	r= 0.81*, p<0.001**	r= 0.76*, p<0.001**	r= 0.852*, p<0.001**						
TSH (mIU/I)	r= 0.272, p>0.05	r= 0.201, p>0.05	r= 0.235, p>0.05	r= 0.189, p>0.05					
FT3 (pg/ml)	r= 0.566*, p<0.001**	r= 0.499*, p<0.01**	r= 0.477*, p<0.01**	r= 0.531*, p<0.01**	r= 0.102, p>0.05				
FT4 (ng/dl)	r= 0.523*, p<0.01**	r= 0.486*, p<0.01**	r= 0.376*, p<0.05**	r= 0.361*, p<0.05**	r= 0.099, p>0.05	r= 0.123, p>0.05			
Trunk / extremities fat <i>ratio</i>	r= 0.588*, p<0.001**	r= 0.384*, p<0.05**	r= 0.56*, p<0.001**	r= 0.463*, p<0.01**	r= 0.175, p>0.05	r=0.586*, p<0.001**	r=0.512*, p<0.01**		
Leptin	r= 0.042, p>0.05	r=0.101, p>0.05	r=0.097, p>0.05	r=0.013, p>0.05	r=0.247, p>0.05	r=0.609*, p<0.001**	r=0.57*, p<0.001**	r=0.197, p>0.05	
		GR	OUP B AFTER	COMPLETE WE	IGHT RESTORA	TION			
	Total body fat mass (%)	Total body fat mass (Kg)	Trunk fat mass (%)	Trunk fat mass (Kg)	TSH (mIU/I)	FT3 (pg/ml)	FT4 (ng/dl)	Trunk / extremities fat <i>ratio</i>	Leptin
Total body fat mass (%)									
Total body fat mass (Kg)	r= 0.674*, p<0.001**								
Trunk fat mass (%)	r= 0.389*, p=0.05	r= 0.025 p>0.05							
Trunk fat mass (Kg)	r= 0.357*, p<0.05**	r= 0.499* p=0.011**	r= 0.398*, p=0.049**						
TSH (mIU/I)	r= 0.221, p>0.05	r= 0.188, p>0.05	r= 0.191, p>0.05	r= 0.127, p>0.05					
FT3 (pg/ml)	r= 0.309*, p<0.05**	r= 0.356*, p<0.05**	r= 0.501*, p<0.01**	r= 0.442*, p<0.01**	r= 0.156, p>0.05				
FT4 (ng/dl)	r= 0.395*, p<0.05**	r= 0.323*, p<0.05**	r= 0.333*, p<0.05**	r= 0.385*, p<0.05**	r= 0.127, p>0.05	r= 0.131, p>0.05			
Trunk / extremities fat <i>ratio</i>	r= 0.218, p>0.05	r= 0.191, p>0.05	r= 0.375*, p<0.05**	r= 0.288 p>0.05	r= 0.191 p>0.05	r= 0.289 p>0.05	r= 0.289 p>0.05		
Leptin	r= 0.045, p>0.05	r= 0.021, p>0.05	r= 0.168, p>0.05	r= 0.135, p>0.05	r= 0.177, p>0.05	r= 0.59*, p<0.001**	r= 0.511*, p<0.01**	r= 0.158, p>0.05	
TSH: thyroid-stimulating hormone ET3: free trijodothyronine. ET4: free thyroxine: * Pearson's statistically significant correlation coefficient ( $r > 0.3$ ); ** Statistically significant difference ( $p < 0.5$ )									

Table 3 Pearson's correlation coefficient analysis after complete weight restoration in Group A and Group B.

program and on complete weight restoration, adolescents from both groups showed normal FT3, FT4 and TSH values, which, in each group considered separately, were statistically significantly higher than at the time of first attendance. Finally, at the same time point, the Group A adolescents showed statistically significantly higher levels of FT3, FT4 and TSH compared with the Group B girls.

Several hormones and peptides have been shown to affect thyroid hormones in adolescents with AN. A keynote hormone in AN, especially in girls who suffer from amenorrhea, is leptin. In these girls, leptin has been found correlate positively with FT3 and FT4, while exogenous leptin administration has been shown to increase levels of thyroid hormones in adolescents with hypothalamic amenorrhea <sup>[19-21]</sup>. This was also shown in our study. In both groups, leptin levels were statistically significantly lower at first attendance than at the time of complete weight restoration, and statistically significantly higher in adolescents who recovered their menses compared with girls who remained amenorrheic despite achieving weight restoration. Leptin levels were also positively correlated, to a

statistically significant degree, with FT3 and FT4, but not with TSH, at the time of complete weight restoration, not only in Group A, but also in the Group B adolescents.

Even though weight gain and normalization of BMI is known to restore normal thyroid hormone levels, there is no known study that has examined the role, in thyroid hormone abnormalities, of body composition and fat distribution in specific body regions. The first finding of our study was that although the Group A and Group B girls showed no statistically significant difference in BMI, total body fat mass (Kg and %), trunk fat mass (Kg and %), free fat mass (Kg and %), and trunk/ extremities fat ratio at first attendance, at the time of complete weight restoration, the Group A adolescents showed statistically significantly higher total body fat mass (Kg and %) and trunk fat mass (Kg and %) levels, as well as a significantly higher trunk/ extremities fat *ratio*, while the Group B girls had statistically significantly higher free fat mass levels (Kg and %). In both groups, no statistically significant difference change in BMI was found at the end of the study. Unsurprisingly, the Group A adolescents, who recovered their menses, showed higher levels of fat mass, and fat distribution in the trunk, but lower levels of free fat mass, while the weight increase in the Group B girls tended to be attributable to free fat mass, rather than total body fat mass. Second, in both groups, a statistically significantly positive correlation was found between FT3, FT4 and total body fat mass (Kg and %) and trunk fat mass (Kg and %) at the time of complete weight restoration, but no correlation between TSH and total body fat mass (Kg and %) or trunk fat mass (Kg and %) was found in either group at this time point. It is important to note that, at this time point, the adolescents who regained their menses showed a statistically significantly positive correlation between FT3, FT4 and trunk/extremities fat ratio, but not between TSH and trunk/extremities fat ratio, in contrast with the girls with persistent amenorrhea, who did not present a statistically significant positive correlation between FT3, FT4 or TSH and trunk/extremities fat ratio at this time.

#### Conclusion

Adolescents with AN are known to present "sick euthyroid syndrome", characterized by decreased levels of FT3 and FT4, and usually normal TSH levels (even though decreased TSH levels have been reported in some girls with AN). Weight recovery leads to normalization of thyroid hormones in these adolescents. We used the PubMed database as a primary source for this research, which appears to be the first known study showing a statistically significantly positive correlation between total body fat mass (Kg and %), trunk fat mass (Kg and %) and FT3 and FT4 levels in adolescents with AN who achieved complete weight restoration (with or without recovery of menses). It also seems to be the first known study to find that distribution of fat mass in the trunk (as expressed by trunk/extremities fat ratio) was statistically significantly positively correlated with FT3 and FT4 in girls who recovered their menses, in contrast with adolescents who remained amenorrheic. Further studies, with larger samples, are needed in order to strengthen these results.

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#### Impact of levonorgestrel-releasing intrauterine system on levels of serum hemoglobin and ferritin in women with a normal and elevated body mass: 1-year follow-up

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#### ABSTRACT

**Objective:** To explore the relationship between overweight and iron status and identify the prevalence and nature of iron deficiency (ID) in a cohort of young healthy overweight and normal weight women using the levonorgestrel-releasing intrauterine system (LNG IUS) as a contraceptive method.

Design: Prospective non-randomized open-label trial.

Sample: 33 women who wanted to use the LNG IUS for contraception.

**Methods:** The participants were divided into 2 groups according to their body mass index (BMI) at the beginning of the research: 19 women formed the LNG-IUS I group (BMI  $\leq$ 25); 14 women were included in the LNG-IUS II group (BMI $\geq$ 25). The women were also analyzed according to levels of serum hemoglobin: >120 g/L (non-anemic) and <120 g/L (anemic) and serum ferritin: <15ng/mL (iron deficiency) and >15 ng/mL.

**Results:** At the beginning of the research, 12 women (22.2%) were diagnosed with anemia and 15 women (27.8%) with severe iron deficiency (<15 ng/mL). After six months of using the contraception, statistically significant increases in S-Hb were found in both S-Hb <120 subgroups (+20.2; p=0.01 and +21.1; p< 0.05 g/L), respectively. Instead, S-Fe increased in the LNG-IUS I group (+17.58; p=0.01 ng/L) but decreased in the LNG-IUS II group (-8.45; p=0.50 ng/L).

After 12 months' use of the contraceptive method, S-Fe increased by +11.00; p=0.11 ng/mL in the LNG-IUS I group, but decreased by -1.07; p=0.3 ng/mL in the LNG-IUS II group.

**Conclusions:** There are various possible reasons for iron deficiency anemia among women with different BMIs, and it cannot be explained only by menstrual bleeding patterns. Our study shows that, among women who chose the LNG IUS for contraception, serum hemoglobin and ferritin levels increased faster in the group with a normal body mass than in the group with elevated body mass.

#### **KEYWORDS**

Levonorgestrel-releasing intrauterine system, serum hemoglobin, serum ferritin, contraception, anemia, iron deficiency.

#### Introduction

The levonorgestrel-releasing intrauterine system (LNG IUS) has been used for contraception in Europe since 1990<sup>[1]</sup>. The LNG IUS also significantly reduces menstrual bleeding <sup>[3]</sup>, which is particularly important for women with menorrhagia – pathological blood loss during the menstrual period (over 80 ml). In these cases, the LNG IUS normalizes menstrual bleeding <sup>[3-5]</sup>, thus restoring the iron reserves in the body and the level of serum hemoglobin (S-Hb) <sup>[5]</sup>.

The LNG-IUS has been assessed for its effect on menstrual blood loss and can work as an alternative to hysterectomy <sup>[3,6-10]</sup>. Menstrual blood loss with the LNG-IUS dropped by 86% at 3 months, and 97% at 6 months. It was observed that parameters of anemia, such as hematocrit and ferritin levels, improved <sup>[9]</sup>.

Globally, the two most widespread nutritional disorders are obesity and iron deficiency (ID). ID and ID anemia (IDA) are major public health issues <sup>[10]</sup>; both are more observed in developing countires, although decreased iron levels are also found in developed countries. Epidemiological evaluation shows that

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#### Abbreviations

LNG IUS: Levonorgestrel-releasing intrauterine system; S-Hb: Serum hemoglobin; S-Fe: Serum ferritin; ID: Iron deficiency; IDA: Iron deficiency anemia

anemia is more frequent with age <sup>[11]</sup>. Previous studies show a link between ID and obesity in children and adults <sup>[12,13]</sup>. A decreased level of serum iron was found with weight gain and increased body mass index (BMI).

Worldwide, reproductive age women face a risk of IDA, which causes morbidity and mortality <sup>[14]</sup>. This could be connected with higher parity, which reduces iron levels in these women <sup>[15]</sup>.



There is a tendency for elevated hemoglobin and ferritin concentrations in obese populations. Previous studies have identified a link between ID and obesity <sup>[15]</sup>.

Although many studies have evaluated the impact of the LNG IUS on anemia indicators, these studies offer only limited insight into changes in anemia indicators in relation to BMI. Our study aimed to identify the correlation between overweight and iron status, and to determine the prevalence and nature of ID in a cohort of young healthy overweight and normal weight women using the LNG-IUS as a method of contraception.

#### **Materials and Methods**

Women aged 18-45 years attending I. Vasaraudze's Private Clinic Ltd. from 1 January 2012 to 31 December 2013 to be counseled on contraception were invited to participate in a prospective non-randomized open-label research study. The women themselves chose a contraceptive method, and the ones who chose the LNG-IUS were included in the study. They were divided into two groups: LNG-IUS with BMI  $\leq$  25 (LNG-IUS I group) and LNG-IUS with BMI  $\geq$  25 (LNG-IUS II group).

According to their S-Hb and serum ferritin (S-Fe) levels at the start of the study, the women were further divided into 4 subgroups: S-Hb < 120 and  $\geq$  120 g/L; S-Fe < 15 and S-Fe  $\geq$  15 ng/mL. When choosing the contraceptive method, the respective contraindications were considered. The goal was to recruit at least ten women in each group, or as many women as possible until the end of the recruitment phase.

Women who met the criteria gave their informed consent before they were included in the sample; the women started to use the hormonal contraception method in accordance with the instructions of the manufacturer. When starting the study, all women were measured, without footwear, for body mass and height. Height was approximated to the nearest 0.1 cm, and weight was approximated to the nearest 0.1 kg.

They were examined at the beginning of the study, after six months and after twelve months. Blood samples for testing were collected a fasting period of 8–12 hours via venipuncture.

#### **Statistical analysis**

The IBM SPSS v.23. program was used. Data were presented as mean values and standard deviation (mean  $\pm$  SD) for continuous variables, and as counts and percentages [%] for categorical variables. Comparisons were made using an independent samples t-test. Cohen's d was used to calculate the effect size (>0.8 = large, 0.8–0.3 = medium, and <0.3 = small). The relationships between variables were evaluated using the Spearman-Rank correlation coefficient (rs). All tests were considered statistically significant at p<0.05.

As no statistical differences between indicators were observed based on p values, Cohen's d values were calculated to evaluate effect sizes. However, a big statistical effect does not always mean a big clinical difference between indicators.

The research had been approved by the Riga Stradins University ethics committee on 26 September 2013.

#### **Results**

454 women who attended the clinic from 1 January 2012 to 31 December 2013 were invited to participate in the research. Of these, 141 (31.1%) agreed and underwent the examinations necessary to be included in the research; 33 women (23.4%) underwent all the necessary examinations and met the inclusion criteria. After being included, the women were divided into two groups according to their BMI at the beginning of the research: 19 women (57.6%) were included in the LNG-IUS I group; and 14 (42.4%) in the LNG-IUS II group. During the research, 2 women (6.0%) were excluded from the study groups because they had side effects and stopped using the contraception. The baseline characteristics of the study groups are summarized in Table 1.

At the beginning of the research, the average level of S-Hb in the LNG-IUS I group was  $126.15\pm9.7$  g/L, versus  $126.8\pm12.78$  g/L in the LNG-IUS II group, without a statistically significant difference between the groups (p=0.56); after the women had been using the LNG-IUS for 6 months, S-Hb was  $133.95\pm9.28$  g/L in the first group and  $132.43\pm9.66$  g/L in the second group (p=0.64); after they had been using it for 12 months, the levels were  $132.16\pm8.91$  and  $134.71\pm71$  g/L (p=0.5), respectively (Fig. 1). After 6 months of LNG-IUS use, S-Hb reached its plateau in the first group, whereas in the second group it continued to increase beyond 6 months, as shown

Table 1 Baseline characteristics of women who participated in the study (N=33)

	LNG-IUS I (N=19)	LNG-IUS II (N=14)	p-value	Cohen's d
Age (years)	33.95±6.15 (23-44)	36.21±4.44 (29-44)	0.25	0.43
BMI (kg/m²)	22.10±2.14 (18.00-24.90)	28.00±2.85 (25.3-35.6)	<0.001	2.38
Deliveries	1.74±0.56 (0-3)	1.50±1.09 (0-3)	0.42	0.27





Group	Parameter		Baseline	6 months	12 months
		S-Hb<120	113.00±5.52 (107-119)	133.20±11.10 (119-147)	127.60±9.90 (115-141)
	S-Hb (g/L)	S-Hb≥120	130.85±5.51 (122-137)	134.21±9.00 (122-151)	133.78±8.27 (116-148)
		p-value	<0.001	0.84	0.20
		Cohen's d	3.10	0.10	0.66
LNG- 105 I		S-Hb<120	11.06±5.44 (5.40-19.00)	28.64±21.30 (11.30-63.10)	39.64±15.07 (24.70-61.20)
	S-Fe (ng/mL)	S-Hb≥120	42.54±24.51 (8.40-90.50)	48.72±18.45 (8.40-73.20)	50.45±25.48 (8.40-96.10)
		p-value	0.01	0.04	0.39
		Cohen's d	1.77	1.00	0.52
	S-Hb (g/L)	S-Hb<120	113.00±8.83 (100.00-119.00)	134.00±12.93 (124-153)	132.00±15.12 (117-153)
		S-Hb≥120	132.40±9.51 (123-149)	131.80±8.80 (120-150)	135.80±10.04 (125-152)
		p-value	0.004	0.71	0.58
		Cohen's d	2.11	0.18	0.23
LNG- IUS II		S-Hb<120	24.57±25.36 (2.40-54.70)	16.12±14.52 (2.4-35.20)	15.05±13.05 (2.40-31.50)
	S-Fe (ng/mL)	S-Hb≥120	35.17±26.03 (8.90-89.80)	47.11±37.11 (22.20-132.00)	49.01±31.48 (10.60-102.00)
		p-value	0.50	0.04	0.01
		Cohen's d	0.41	1.10	1.43

Table 2 Levels of serum hemoglobin (g/L) and serum ferritin (S-Fe) in the research groups according to baseline levels of serum hemoglobin

by the level after 12 months of use. Tables 2 and 3 summarize the S-Hb and S-Fe levels recorded in the study groups divided according to different baseline levels of S-Hb (Table 2) and S-Fe (Table 3). At the beginning of the research, the average level of S-Fe was  $34.25\pm24.94$  ng/mL in the LNG-IUS I group and  $32.14\pm25.34$  ng/mL in the LNG-IUS II group, with no statistically significant difference between the groups (p=0.98). After 6 months, the average level of S-Fe was  $43.44\pm20.71$  in group I and  $38.26\pm34.84$  in group II (p=0.91). After 12 months, the average level of S-Fe was  $47.61 \pm 23.31$  in group I and  $39.31\pm31.29$  in group II (p=0.3) (Fig.2). S-Fe did not reach its plateau level in either of the groups and continued to increase also after 12 months of use.

In the LNG-IUS II, S-Hb<120 subgroup, S-Fe level decreased from 24.57±25.36 ng/mL to 16.12±14.52 after six months' use of the contraception; after 12 months, it was found to have decreased further, to 15.05±13.05 ng/mL (Table 2).

After exploring the possibility of correlations between BMI, age, S-Hb and S-Fe, it was found that there was no statically significant correlation (p>0.05).





Group	Parameter		Baseline	6 months	12 months
LNG- IUS I	S-Hb (g/L)	S-Fe<15	116.16±8.23 (107-126)	137.16±7.65 (127-147)	131.00±9.12 (115-141)
		S-Fe≥15	130.77±6.31 (119-137)	132.46±9.86 (119-151)	132.70±9.14 (116-148)
		p-value	<0.001	0.31	0.71
		Cohen's d	1.97	0.53	0.11
	S-Fe (ng/mL)	S-Fe<15	16.83±19.54 (5.40-56.30)	31.60±21.75 (8.40-63.10)	35.00±18.74 (8.40-61.20)
		S-Fe≥15	42.30±24.16 (17.90-90.50)	48.90±18.54 (12.20-73.20)	53.42±23.51 (17.90-96.10)
		p-value	0.03	0.09	0.11
		Cohen's d	1.16	0.8	0.86
LNG- IUS II	S-Hb (g/L)	S-Fe<15	119.75±15.67 (100-138)	126.75±4.57 (120-130)	127.75±2.06 (125-130)
		S-Fe≥15	129.70±11.07 (115-149)	134.70±10.38 (124-153)	137.50±12.25 (117-153)
		p-value	0.20	0.17	0.03
		Cohen's d	0.91	1.00	1.12
	S-Fe (ng/mL)	S-Fe<15	7.25±4.62 (2.40-12.90)	16.77±13.71 (2.40-28.60)	25.32±25.24 (2.40-56.10)
		S-Fe≥15	42.10±23.13 (11.40-89.80)	46.85±37.45 (19.20-132.00)	44.90±32.86 (10.60-102.00)
		p-value	0.01	0.04	0.30
		Cohen's d	2.10	1.08	0.70

Table 3 Levels of serum hemoglobin (g/L) and serum ferritin (ng/mL) in the research groups divided according to baseline ferritin levels

#### **Discussion**

As excessive menstrual bleeding is the most frequent cause of anemia in women of reproductive age, the considerable reduction in the menstrual bleeding that is observed in LNG-IUS users naturally leads to a long-term increase in their levels of S-Hb and S-Fe<sup>[16]</sup>. ID in the absence of anemia adversely affects physical performance, mental health and cognitive function<sup>[17]</sup>.

The results of our study show that moderate ID with reduced S-Fe level for women with elevated BMI is the most common iron-related abnormality (27.8%). During the use of the contraception, a significant increase in the level of S-Hb was observed in the group of women with a normal BMI; by contrast, in the group of women with elevated BMI, significant changes were observed only after 12 months. This indicates a very gradual correction of ID. Despite this, even after using the LNG-IUS for 12 months, 21.4% of women with increased BMI had ID. Our research shows that the treatment of IDA and ID may require a longer time than that required for the correction of menstrual bleeding patterns. Obesity is a worldwide pandemic, while ID is the most widespread single microelement deficiency.

Estrogen is important for regulating iron metabolism, cardiovascular circulatory system, skeletal muscle system, central nervous system, bacterial infections, and estrogen-related diseases <sup>[18]</sup>. Premenopausal obese and overweight women had significantly lower estradiol levels, independently of age, race and smoking habits <sup>[19]</sup>.

In the female body, serum iron storage is closely linked to estrogen level. A study by Qian et al. showed that estrogen is involved in regulating ferroportin expression <sup>[20]</sup>. Ferroportin and hepcidin are critical proteins for the regulation of systemic iron homeostasis. They found that transcription of hepcidin was reduced by estrodiol treatment. In young women, hepcidin inhibitionby high estrogen increases iron uptake, in order to compensate for iron loss during menstruation. This mechanism also plays a role in increased iron reserves in young women who use oral contraceptives. These authors' results show that estrogen deficiency could not lead to iron increase. Previous studies have shown evidence of regulatory effects of estrogen on iron metabolism, but future studies are still necessary to clarify, in detail, the mechanisms involved.

Many studies <sup>[21-25]</sup> show that iron plays a role in pathogenesis of many diseases, such as ischemic heart disease, cancer, diabetes, infections and neurodegenerative disorders. Healthy levels of iron have not yet been standardized, but it is likely that ID or iron overload could cause adverse health effects. Jian et al. <sup>[20]</sup> reported that ID in young women contributes to high breast cancer recurrence, while increased iron plays a role in high breast cancer incidence in postmenopausal women.

It has been shown in the literature that elevated c-reactive protein and low median hepcidin can detect a greater percentage of participants with ID<sup>[26]</sup>. Previous studies did not establish a relationship between BMI and Fe status. Obese women had lower Fe absorption when compared with overweight women. This may be due to subclinical inflammation associated with obesity. Previous studies have concluded that obesity is significantly related to ID<sup>[27]</sup>.

Mean and median hemoglobin levels have been found to be significantly higher in abdominally obese compared with totally obese women. The mean hemoglobin level was positively and significantly associated with waist circumference and negatively and insignificantly associated with BMI. Overweight women reported greater ID than normal weight women. These findings show that overweight females are at greater risk of ID and that inflammation caused by excess adipose tissue plays a role in this phenomenon <sup>[25,26]</sup>.

On the other hand, previous studies <sup>[28-35]</sup> also show higher hemoglobin and ferritin concentrations and lower transferrin saturation in overweight women. Thus, future studies involving inflammatory cytokines, soluble transferrin receptors and hepcidin are necessary to confirm the impact of obesity on iron metabolism.

Serum ferritin, which is a marker of iron metabolism, is considered a biomarker of chronic low-grade inflammation. After menopause, there are significant increases in insulin resistance (IR) and metabolic syndrome (MetS), which are very often considered inflammatory conditions. Serum ferritin levels were positively and independently associated with IR and MetS in postmenopausal women. S-Fe levels in postmenopausal women could help to identify the presence of IR and MetS <sup>[30]</sup>.

However, there have been studies showing that obese and normal weight persons do not differ in total daily iron consumption. At the same time, the fat mass is regarded as a major negative predictor of serum iron level. Furthermore, a connection is reported between increased BMI and Hb, as well as high S-Fe levels<sup>[31]</sup>.

A number of age-related, dietary and inflammatory-associated factors influence iron levels in overweight young women. ID and IDA could lead to exhaustion, thus reducing physical activity and further contributing to weight gain. ID and obesity are not just two prevalent conditions but are also molecularly linked and mutually affect each other <sup>[32]</sup>.

Our results show that ID, as reflected by low ferritin, is still the major iron-related abnormality among overweight women. Our study also emphasizes the importance of taking into account simple ID in healthy overweight young women using LNG-IUS for contraception.

#### Conclusions

There are various possible reasons for IDA among women with different BMIs, and it cannot be explained only by menstrual bleeding patterns. Among women who chose the LNG-IUS for contraception, we found that S-Hb and S-Fe levels increased faster in those with a normal BMI compared with the group of women with elevated body mass.

This research shows that even women without menstrual bleeding complaints should be evaluated and, if necessary, appropriately treated for IDA before starting contraceptive use of the LNG-IUS.

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#### Rationale and development of the *Vita Nova* project: and app and service to reduce cardiovascular and metabolic risks in pre-menopausal and menopausal women

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#### ABSTRACT

**Background and purpose:** Despite the recent increase in research and development of mobile self-care tools, there is still a marked lack of solutions focusing on prevention of the negative health effects of the menopause. Moreover, most of the solutions that do exist are not based on well-founded user models, such as personas, and fail to exploit the potential of persuasive mobile technology, and thus result in a user experience that is neither engaging nor adaptive.

**Methods:** We describe how we designed personas during the development of a mobile application for menopause selfcare. We applied the principles of the Persuasive Systems Design model and the Just-in-Time Adaptive Interventions framework, together with participatory techniques and demographic data analysis.

**Results:** The *Vita Nova* App prototype has been successfully completed. The usability of this mobile app and service, designed to accompany and coach women regarding the menopause, automatically adapting to their wants and needs in order to induce positive health-related behavioural changes, is currently being verified through a pilot study in a "real-life" scenario of a small group of healthy peri-menopausal and early- and late- postmenopausal women. This preliminary investigation is now in its final stages.

**Conclusions:** This approach allowed us to come up with reliable representations of our target users and their goals, which in turn enables us to better define and communicate our project's scope and features. Moreover, this approach is not limited to the menopause domain. In the future, it could also be used, to reliably represent users, when designing mobile self-care solutions in other health-related domains or scenarios of female life.

Further investigations in larger study populations of healthy peri- and post-menopausal women will be mandatory in order to assess the real impact of this app

#### **KEYWORDS**

Vita Nova App; self-care application; m-Health; e-Health; cardiovascular risk; menopause; prevention; metabolic.

#### Introduction

The world's population is aging at a historic rate, and this is seen particularly in the most economically developed countries. This demographic shift implies significant changes at both societal and economic level, and has already started to have a negative impact on public healthcare systems. For these reasons, there has emerged a growing interest in healthy aging and personal self-care methods, especially ones based on Information and Communication Technology (ICT). The plethora of available health and fitness smartphone applications (apps), as well as related research initiatives, are a prime example of this phenomenon. However, most of these apps are merely trackers that do not adapt to users' health status and behaviour, and many do not apply best practices on human-computer interaction (HCI); both of these factors result in poor user adherence. These issues are particularly true of the few available apps (among those aimed at women) that focus on the menopause and its effects <sup>[1]</sup>.

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The symptoms of the menopause can be distressing, particularly as they occur at a time of life that sees women playing an important social role within both the home (family) and the workplace. The hormonal changes that begin during the menopausal transition affect many biological systems. For instance, the signs and symptoms of menopause include central nervous system-related disorders, metabolic, weight, cardiovascular and musculoskeletal changes, skin and urogenital atrophy, and sexual dysfunction. The physiological basis of these manifes-



tations is emerging as complex and related not only to oestrogen deprivation, but also other factors. New findings, coming mainly from longitudinal population studies, show that ethnic, geographical and individual factors affect symptom prevalence and severity. Moreover, and of great importance from a clinical practice perspective, the latest research has highlighted that certain menopausal symptoms can be associated with the onset of other disorders, and might therefore serve as predictors of future health risks in postmenopausal women <sup>[2]</sup>.

In the post-menopause, there emerge long-term manifestations of definitive oestrogen deprivation; for example, urogenital atrophy, skin ageing, and osteoporosis might develop during this time. In addition, a shift towards central body fat distribution, and consequent metabolic alterations, might occur as a result of an increased androgen to oestrogen *ratio*, which is also driven by increased insulin resistance <sup>[3]</sup>.

One of the main complaints from women at midlife is increased weight. Indeed, the prevalence of obesity is higher in postmenopausal women than in premenopausal women. Absolute weight gain in women at midlife seems to be fundamentally related to ageing rather than to the menopause itself<sup>[4]</sup>. In women aged 40-55 years, the average weight gain was reported by one study to be 2.1 kilograms over 3 years <sup>[5]</sup>. On the other hand, a phenomenon that does seem to be menopause-dependent is redistribution of body fat; this is characterized by accumulation of mostly visceral adiposity at the trunk, leading to an increase in waist circumference and an obvious change in body shape <sup>[6]</sup>. Visceral adipose tissue poses a greater health risk than subcutaneous fat and, in general, is an independent cause of cardiovascular disease (CVD), primarily due to an increase in insulin resistance, and the consequent risk of developing diabetes mellitus and metabolic syndrome. Furthermore, cross-sectional and longitudinal studies have provided evidence that ovarian failure is causative of visceral fat accumulation during menopause [7-9].

In women, atherosclerosis and the risk of cardiovascular adverse events increase after the menopause; this might be due in part to the production of pro-inflammatory cytokines and adipokines in visceral adipose tissue. Increased deposition of visceral fat in postmenopausal women might be associated with fat accumulation in other visceral tissues, such as the heart <sup>[10]</sup>. Indeed, it has been reported that late peri-menopausal and postmenopausal women have markedly greater volumes of heart fat compared with premenopausal women independent of age, race, obesity, or other covariates [11]. The markedly reduced exposure to oestrogen during the menopause might have a negative effect on endothelial cell growth and reduce the inhibitory effect of female sex hormones on the growth and proliferation of vascular smooth muscle cells. Moreover, although blood pressure levels seem to be lower, on average, in premenopausal women than in their male counterparts, this advantage is lost around the time of the menopause. At this time, blood pressure levels start to rise in women, reaching levels similar to those of men of the same age group. These negative changes in cardiovascular features increase the risk of adverse cardiovascular events [12,13].

An emerging concept is that some menopausal symptoms might be predictive of future health complications. Indeed, se-

vere vasomotor symptomatology and poor quality of sleep are associated with an increased risk of CVD and postmenopausal depression. Furthermore, depressive symptoms, vasomotor symptoms and sleep disorders might increase susceptibility to developing cognitive dysfunction, while severe hot flushes have been associated with an increased risk of osteoporosis and bone fracture. Finally, as menopause seems to accelerate the ageing process, it is conceivable that, in addition to loss of ovarian function, the manifestation of menopausal symptoms might be in part due to ageing <sup>[3]</sup>.

The large number of studies performed over the past decade in women transitioning through menopause highlights the need to closely monitor health parameters at this stage of life, and promote a healthy lifestyle. Appropriate healthcare and lifestyle changes should start before the menopausal transition, in order to counteract the emergent cardiovascular risk factors and possibly reduce bothersome symptomatology. It is also important for medical practitioners to consider a woman's home and work environment and her ethnicity, as these factors, too, will profoundly affect her experience as she goes through the menopause. Although the management of symptoms through pharmacological or cognitive-behavioural therapy approaches might improve women's quality of life in the short-term, further research is needed in order to develop new strategies to attenuate long-term health risks that are often intensified by the loss of ovarian function.

Midlife is a time of profound personal and social change for women. The perception and interpretation of menopausal symptoms, and therefore their interference with day-to-day life, are influenced by social and cultural beliefs. At international level, ~20% of women perceive menopause as a disease, even without necessarily being fully aware of its symptoms and health implications [14]. Depending on personal and work characteristics, even mild menopausal symptoms can be distressing for some women, and most women with pervasive menopausal symptoms will experience profound difficulty in coping with daily life. The personal experience of menopausal symptoms, particularly bodily changes related to ageing and the awareness of the loss of fertility, may alter self-image. Life events might produce a change of role and/or identity around the time of the menopausal transition. The 'empty nest syndrome', retirement from work, having frail or ill parents, as well as the loss of a parent or partner are all circumstances that frequently occur at midlife. These new circumstances may imply depleted personal and social networks, a feeling of being relegated to a less prestigious status, and an increase in caregiving activities, with an overall decline in quality of life [14,15]. Thus, midlife and the menopausal transition are often perceived as a time of crisis by women and the presence of distressing menopausal symptoms adds to the perception of deteriorating mental and physical wellbeing, which has indirect consequences on health <sup>[16]</sup>. Nonetheless, many women instead perceive the menopause as a natural phase of life without negative implications <sup>[17]</sup>. The reasons for this interpersonal variability in the perception of the menopause might be attributable to the relative intensity of symptoms, but it might also depend on how women interpret and manage these symptoms according to their social position and cultural inclination [18,19].

Approximately 30–40% of women report that menopausal symptoms reduce their performance in the workplace, with the most disruptive symptoms being hot flushes, insomnia, a feeling of tiredness and poor concentration <sup>[19]</sup>. Even women who are not heavily burdened by menopausal symptoms, often pay a price in the form of a perception of reduced social desirability and feelings of shame or embarrassment, sometimes prompted by unwelcome comments from colleagues <sup>[20-22]</sup>. Targeted strategies aimed at making the menopausal transition and its symptoms recognised socially and accepted in the workplace <sup>[18,23,15]</sup> — such as promoting self-help reading matter, specific training, flexible working hours or shift changes, reviews of workplace ventilation and temperature — have proven valuable in helping women to share their experiences with peers, and together seek solutions and develop coping strategies.

However, many women do not appreciate the need to improve their health-related behaviour and lifestyle in order to reduce the negative effects of the menopause. Inspired by the increasing prevalence of smartphones across the general population, we think that an adaptive and personalised app that empowers women regarding their menopausal transition would be a fitting solution to this self-care necessity. This is the main rationale behind the ongoing *Vita Nova* project.

#### Methods

The *Vita Nova* project consists of the development of a mobile app and service to accompany and coach women regarding their menopause; adaptable to their wants and needs, it is intended to promote health-related behavioural changes. The service's primary goal is to reduce the higher cardiovascular risk caused by the menopausal transition. The project's consortium members are all based in the Italian Region of Tuscany, and have different backgrounds and areas of expertise: three private companies (business and ICT services), a public research organisation (data modelling and HCI research), a public university (experimental gynaecology and socioeconomics research), and an external consultant (psychology). From the very beginning, the consortium decided to utilise a user-centred design, with the intention of increasing user adherence to our health-related app.

The spread of pervasive and ubiquitous computing (e.g. smartphones, wearable devices, domestic sensors) has spurred the development of mobile health technology and solutions for self-care. Nevertheless, available mobile self-care solutions focusing specifically on the menopausal transition are scarce, as is the respective scientific literature. Among the few available apps present in the scientific literature we found MenoPro, developed by the North American Menopause Society. Meno-Pro helps gynaecology clinicians decide whether their patients should undergo pharmacological treatment for menopause-related symptoms. In addition to the clinician mode, MenoPro also has a patient mode that can be used by women interested in undertaking a treatment, but this mode has very limited features. There is no self-monitoring over time, it is only for women over 45 years old, and behavioural changes are not taken into account<sup>[1]</sup>.

We therefore decided to follow the just-in-time adaptive interventions (JITAI) conceptual framework, which is specific for the design of smartphone solutions that promote health-related behavioural changes based on individual characteristics <sup>[24]</sup>. JITAI revolves around one or more distal outcomes (targeted behaviours), which in turn are connected to a larger set of short-term proximal outcomes, adapted to the framework's four key components: decision points (when), intervention options (what), tailoring variables (whom), and decision rules (how) (Fig.1). Decision points can be expert-specified or user initiated. The choice of intervention options should be based primarily on the targeted proximal outcomes, as interventions vary in terms of kind of support, source, and delivery mode. Tailoring variables describe the user's health status and behaviour, while decision rules are "if-then" statements, used to decide which intervention options to provide based on the values of a given set of tailoring variables. JITAI has been used successfully in health-related apps for the reduction of depression and anxiety <sup>[25]</sup>, sedentariness <sup>[26]</sup>, and addictive behaviour <sup>[27]</sup>. In the case of Vita Nova, the primary distal goal is to reduce the menopause-related cardiovascular risk, by targeting four specific behaviours: physical activity, diet, alcohol consump-

**Figure 1** Main components of the just-in-time adaptive interventions (JITAI) framework.



tion, and smoking. Each of these behaviours has distal goals (e.g., regularly do physical activity, quit smoking) and related proximal goals (e.g. increase number of steps per week, reduce smoking by one cigarette per day).

To this end, our first step consisted of knowledge acquisition and the definition of a representation of women's health status and behaviour. This was done together with experts from the aforementioned consortium members in the bio-clinical, socio-economic, and psychological fields. We also used data from the Italian National Institute for Statistics and Eurostat. Next, and based on the preventive character of Vita Nova, we defined our target users as menopausal and pre-menopausal women aged 40-60 years, who were free of chronic diseases, and had a BMI of no less than 18.5 (underweight) and no more than 30 (obesity). We then elicited the main JITAI tailoring variables based on biological, behavioural, and socio-economic determinants, menopause symptoms, and health-related personality traits. Each variable was selected on the basis of its appropriateness for a mobile app, relevance, measurability, and ease of collection. The proposed interventions are mainly textual recommendations and information, as defined by the aforementioned experts.

A set of 75 tailoring variables has already been defined, some of which can be derived from the user's answer to a single question, while others are derived from their answers to two (e.g. BMI) or more (e.g. personality traits) questions. Given the relatively high number of questions, we also specified a subset of mandatory variables: age, BMI, awareness of having hypercholesterolaemia, awareness of having high blood pressure, smoking status, household composition, household dynamics, living with a partner, and leisure time (hours per week). The remaining variables can be gathered progressively, if applicable (e.g. smoking, menstrual cycle). Table 1 lists all the variables and how they are measured/assessed. Notably, considering that the Vita Nova App is not being developed as a medical device, none of its sections will contain information about or concern menopausal hormonal therapy or drugs, and these topics will not be raised at any point during the interaction with users.

#### Results

The prototype of *Vita Nova* App has been successfully completed, and we have already started "real-life" testing of

Table 1 Complete list of clinical, socio-economic and psychological variables for the tailoring of recommendations.

CLINICAL VARIABLES	MEASURE/SCALE	CLINICAL VARIABLES	MEASURE/SCALE
Family history of AMI	yes/no	Consumption of sugary drinks	times/week
Family history of stroke	yes/no	Sweet food consumption	times/week
Family history of hypertension	yes/no	Salt consumption	times/week
Family history of diabetes	yes/no	Consumption of snacks at work	times/week
Family history of colon cancer	yes/no	Ready-made food consumption	times/week
Family history of breast cancer	yes/no	Cured meat consumption	times/week
Family history of osteoporosis	yes/no	Awareness of hypertension	yes/no
Date of birth	date	Awareness of hypercholesterolaemia	yes/no
Weight	kg	Menstrual regularity	yes/no
Height	cm	Heavy menstrual bleeding	yes/no
Menopause onset	age	Hot flushes an inconvenience	Likert 1-5
Menopause onset age	years	Sweats an inconvenience	Likert 1-5
Smoking status	yes/no	Fatigue an inconvenience	Likert 1-5
Smoking	cig-number	Insomnia an inconvenience	Likert 1-5
Beer consumption	ml/week	Vaginal dryness an inconvenience	Likert 1-5
Wine consumption	ml/week	Dyspareunia an inconvenience	Likert 1-5
Hard liquor consumption	ml/week	Genital prolapse an inconvenience	Likert 1-5
Moderate physical activity	min/week	Urinary incontinence an inconvenience	Likert 1-5
Intense physical activity	min/week	Bladder voiding dysfunction an inconvenience	Likert 1-5
Sexual activity	yes/no	Depressed mood an inconvenience	Likert 1-5
Consumption of fruit/vegetables	times/week	Anxiety an inconvenience	Likert 1-5
Consumption of fatty foods	times/week	Irritability an inconvenience	Likert 1-5
Cooking style	type	Mood changes an inconvenience	Likert 1-5
Fish consumption	times/week	Lack of energy an inconvenience	Likert 1-5
Whole meal consumption	times/week	Short-term memory loss an inconvenience	Likert 1-5
Consumption of pulses	times/week	Loss of concentration an inconvenience	Likert 1-5
Sugar consumption	times/week	Musculoskeletal pain an inconvenience	Likert 1-5 (next page)

SOCIO-ECONOMIC VARIABLES	MEASURE/SCALE	SOCIO-ECONOMIC VARIABLES	MEASURE/SCALE
Perception of income level	Likert 1-5	Retired since	years
Household composition	number	Perception of retirement	to state
Living with partner	yes/no	Perception of employment	to state
Household dynamics	to state	Caregiving Duty activities	hours
Educational attainment	to state	Cultural activities	yes/not
Perception of leisure time	Likert 1-5	Cultural activities, list	to state
Children	number	Cultural activities, time	hours
Marital status	yes/no	Aesthetic treatments	to state
Employment status	to state	Network of friends	yes/no
PSYCHOLOGICAL VARIABLES	MEASURE/SCALE		
Self-efficacy	Likert 0-3		
Self-esteem	Likert 0-3		
Readiness to change	VAS 1-10		

this mobile app and service, designed to accompany and coach women regarding the menopause, automatically adapting to their wants and needs in order to induce positive health-related behavioral changes (Figure 1).

A pilot study on a small group of peri-menopausal, and early and late post-menopausal women is ongoing in order to preliminarily assess the usability and the plausible impact of the prototype on women's quality of life and health.

#### Discussion

Menopausal symptoms have a substantial effect on women's quality of life and performance in the workplace; increased awareness of these symptoms and acquisition of coping strategies might help to address this issue. While menopause per se is not associated with weight gain, it leads to an increase in total body fat and a redistribution of body fat from the periphery to the trunk, which results in visceral adiposity. In parallel, abdominal obesity and menopausal oestrogen decline are associated with adverse metabolic changes and a higher risk of developing CVDs with a plausible impact on the global socio-economic scenario. From this perspective, the Vita Nova project, which consists of developing a mobile app and service designed to accompany and coach women regarding the menopause, automatically adapting to their wants and needs in order to induce positive health-related behavioural changes, could help reduce the higher cardiovascular risk inherently associated with the menopausal transition.

Herein we have presented the principles of persuasive systems and JITAIs, and how we used these to define personas during the interaction design process of a menopause self-care app. This approach allowed us to come up with reliable representations of our target users and their goals, which in turn enables us to better delimit define and communicate our project's scope and features. Moreover, this approach is not limited to the menopause domain. In the future, it could also be proposed in other domains or scenarios of female life such as adolescence or the reproductive period. Following the successful design of the prototype of the app and the forthcoming analysis of the results of the pilot study, further investigations in larger study populations of healthy peri- and post-menopausal women will be mandatory in order to assess its real impact.

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