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CLINICAL CHARACTERIZATION AND GENETIC ANALYSIS IN WOMEN WITH PREMATURE OVARIAN INSUFFICIENCY

Artigo científico

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RESUMO

Objetivos: Foram clinicamente avaliadas mulheres com insuficiência ovárica precoce (POI) e analisadas as suas alterações do cariótipo e do gene *FMR1*, assim como a presença de autoimunidade, no sentido de encontrar a sua etiologia e de avaliar as suas repercussões familiares.

Métodos: Um total de quarenta e uma pacientes com POI foram estudadas retrospetivamente, o qual envolveu a recolha de informação clínica (idade da menopausa, apresentação clínica, história familiar de POI, história pessoal de autoimunidade); avaliação hormonal; pesquisa de autoimunidade tiroideia; ultrassonografia pélvica; avaliação de densidade mineral óssea (BMD), extração de DNA e análise do gene *FMR1*.

Resultados: No presente estudo foi identificada a etiologia em 19 (46,3%) pacientes: nove (22%) com doenças autoimunes (5 com tiroidite autoimune, 1 com tiroidite autoimune e psoríase e 3 com doença de Beçhet, diabetes mellitus ou psoríase); oito (20%) com alterações no cariótipo (6 com mosaicismos do cromossoma X, uma com uma deleção e uma com 47,XXX); e duas (4,9%) com pré-mutação do gene *FMR1*.

Conclusão: Face aos resultados foi possível concluir que em mulheres com história familiar de POI é importante a obtenção de cariótipos e a pesquisa de pré-mutação do gene *FMR1* no sentido de ser efetuado um aconselhamento genético e assim limitar a transmissão de Síndrome do X Frágil (FXS) em gerações futuras.

ABSTRACT

Objective: We clinically evaluated women with premature ovarian insufficiency (POI) and analyzed their karyotype, the number of CGG repeats in the *FMR1* gene and the presence of autoimmunity in order to determine an aetiology and its familiar repercussions.

Methods: A total of forty one patients affected by POI were studied retrospectively, which involved gathering clinical data (age at menopause, clinical presentation, family history of POI, personal history of autoimmunity); hormonal evaluation; screening for thyroid autoimmunity; pelvic ultrasonography; bone mineral density (BMD) and DNA extraction assessment of the *FMR1* gene.

Results: The present study identified the aetiology in 19 (46,3%) patients: nine (22%) with autoimmune diseases (5 with autoimmune tiroiditis, 1 with autoimmune tiroiditis and psoriasis and 3 with Beçhet's disease, diabetes mellitus or psoriasis); eight (20%) with karyotipe abnormalities (6 with X chromosome mosaicisms, one with a deletion and one with 47,XXX); and two (4,9%) with *FMR1* premutation.

Conclusion: We suggest that karyotypes and the determination of *FMR1* allelic forms are worth seeking in those with familial POI in order to enable genetic counselling and limiting the transmission of Fragile X Syndrome (FXS) to future generations.

KEYWORDS

Premature ovarian insufficiency, *FMR1* gene, clinical analysis.

ABREVIATION'S LIST

BMD – Bone Mineral Density

DNA – Desoxyribonucleic Acid

E_2 – Estradiol

FMR1 – Fragile X Mental Retardation 1

FSH – Follicle-Stimulating Hormone

FXS - Fragile X Syndrome

ISCN – *International System for Human Cytogenetic Nomenclature*

LH – Luteinizing Hormone

PCR – Polymerase Chain Reaction

POI – Premature Ovarian Failure

WHO – World Health Organization

INTRODUCTION

Premature ovarian insufficiency (POI) is defined by primary amenorrhea or secondary amenorrhea, for at least the duration of 4-6 months, in women under the age of 40 years, opposed to the median age of natural menopause in Caucasian women at 50 ± 1 years [1]. This early loss of ovarian function is associated with elevated levels of gonadotropins (FSH level > 30U/l) and hypoestrogenism [1,2], being estimated to affect 0,01% of women under the age of 20, 0,1% under 30 and 1% under 40 [3,4] and occurring in 10-28% in women with primary amenorrhea and 4-18% of those with secondary amenorrhea [5] .

This premature hypoestrogenism causes premature aging of several tissues, leading to increased risk of osteoporosis, cardiovascular and neurodegenerative diseases [1]. POI may also be associated with infertility/subfertility, decrease in life-expectancy, cognitive dysfunction, sexual dysfunction and psychological effects [3].

Although in most cases, POI is idiopathic, many causes may lead to its development, including autoimmunity, metabolic abnormalities, infections and iatrogenic causes (surgery, chemotherapy) [6,7]. Besides autoimmune diseases or infections, a genetic cause (4-31 %) is thought to be the mainly responsible for POI development and the *Fragile X Mental Retardation 1* (*FMR1*) gene is the most prominent candidate gene [8]. Genetic causes also include X chromosome abnormalities, such as aneuploidies or structural chromosome defects being the most frequent ones deletions, X-autosome translocations and isochromosomes [6].

The *FMR1* gene is located at Xq27.3 and is responsible for the Fragile X Syndrome (FXS), one of the most common causes of mental retardation, characterized by a wide spectrum ranging from autism behaviour, attention deficits, hyperactivity and features' abnormalities, which results from the expansion of the unstable CGG trinucleotide over 200 repeats in the 5'-untranslated region of the gene's first exon [1,8,9]. The premutation status is

characterized for having an amplification of the CGG triplet number between 55-200 [1,8] and was associated with a high risk factor (13-26 %) for women to develop POI [8,10,11], being susceptible to expansion when passed from a carrier to offspring, despite its incomplete penetrance [12]. The normal rage is considered under 40 CGG repeats and an intermediate 'gray zone' between 41-54 repeats [1,13].

In this study, we were able to clinically evaluate 41 women, retrospectively, and to analyze their karyotype, the number of CGG repeats in the *FMR1* gene and the presence of autoimmunity in order to determine an aetiology and its familiar repercussions.

MATERIALS AND METHODS

Clinical Population

A total of forty five patients from Maternidade Bissaya Barreto – Centro Hospitalar Universitário de Coimbra affected by amenorrhea were studied retrospectively, by consultation of their clinical processes, after an informed consent was obtained.

POI was defined by at least 4 months amenorrhea, two FSH levels measured above 30 mUI/mL at an interval of at least one month and a karyotype excluding Turner's syndrome. Were excluded from this work four women: one aged over 40 years at the time of diagnosis, one with Turner's syndrome, a woman with a history of chemotherapy and pelvic radiotherapy and a woman with a history of bilateral oophorectomy.

Forty one of the patients met all the criteria and completed the study, which involved gathering clinical data (age at menopause, clinical presentation, family history of POI, personal history of autoimmunity); hormonal evaluation (measurements of FSH, LH and estradiol (E2)); screening for thyroid autoimmunity; pelvic ultrasonography; bone mineral density (BMD) and DNA extraction assessment of the *FMR1* gene, done in the Laboratório de Citogenética e Genómica da Faculdade de Medicina da Universidade de Coimbra.

Conventional Cytogenetics

Conventional Cytogenetics was performed on synchronized cultures of peripheral blood lymphocytes using the standard procedures of the Laboratório de Citogenética e Genómica da Faculdade de Medicina da Universidade de Coimbra and following the methodologies described in the *Current Protocols in Human Genetics* [14].

GTG banded chromosomes were analyzed with resolution level >550 bands following the *International System for Human Cytogenetic Nomenclature* (ISCN) [15].

In each patient, 10-15 metaphases were analyzed using a microscope from *Nikon Eclipse* (Nikon Instruments Europe B.V., Badhoevedorp, Holland) with a *Cytovision* system (Applied Imaging International Lda, Newcastle upon Tyne, UK).

DNA Extraction

The allelic forms of the *FMR1* gene were studied on genomic DNA extracted from peripheral blood lymphocytes, by the method of extraction in columns (*Jetquick DNA Midid Spin kit – Genomed*, Germany), according to the protocol suggested from the fabricant.

The extracted DNA was quantified and its purity level was determined using the spectrophotometer *Nanodrop 1000* (*Thermo Scientific, USA*).

***FMR1* gene study**

The determination of the number of *FMR1* CGG repeats was done by analysis of fragments obtained by polymerase chain reaction (PCR), using primers C and F specific for *FMR1* gene [8]. The DNA fragment analysis was done using the GeneMapper software version 4.1 in a ABI PRISM 3130 Genetic Analyzer (*Applied Biosystems, California, USA*) to determine its dimension and the number of CGG repeats. The size of the fragments was determined by comparison with the standard molecular weight *ROX 500* (*Applied Biosystems*).

To distinguish between homozygous normal women and women with complete mutation, in the cases where we obtained just one fragment, it was used the AmplideX® *FMR1* PCR technique (*Asuragen, Inc., Austin, USA*), based in the *Triplet Repeat Primed PCR* [16].

RESULTS

Clinical Characteristics

Most of our patients (n=39; 95%) presented with secondary amenorrhea and normal puberty, one (2,5%) presented with primary amenorrhea and normal pubertal development and another (2,5%) displayed primary amenorrhea and pubertal delay. The mean age at the time of the diagnosis of those with secondary amenorrhea was 35,5 ± 4,3 years [23-40]. The figure 1 and 2 show the baseline demographic and clinical characteristics of these patients at diagnosis.

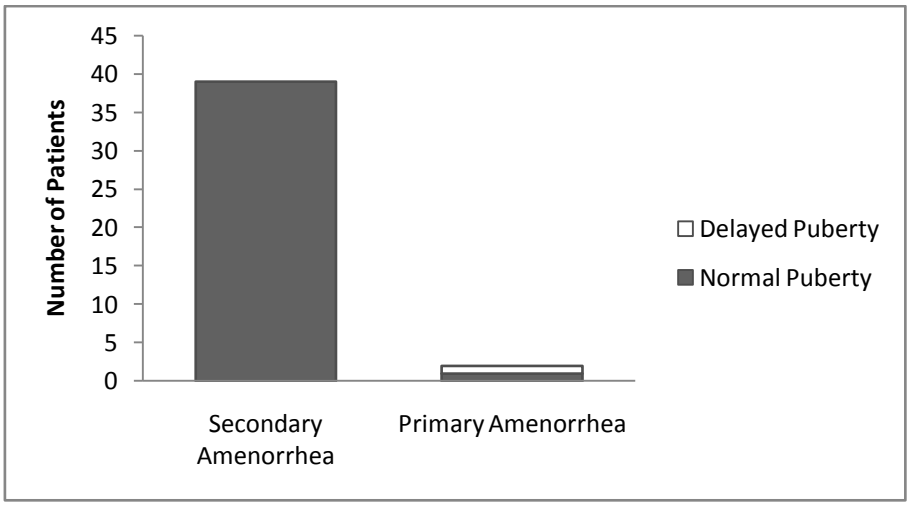

Figure 1: Type of amenorrhea and pubertal development at diagnosis.

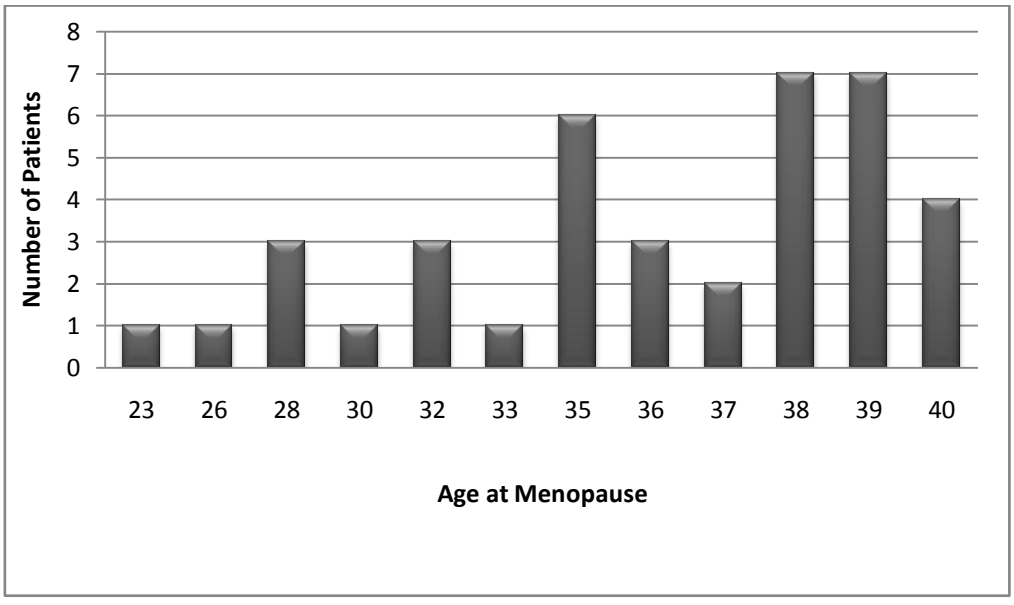

Figure 2: Distribution of POI patients at age of menopause at diagnosis.

Sixteen patients (39%) had a family history of POI, nine with only one relative with history of POI, seven of which involved their mother or sister, one a maternal aunt and one a paternal aunt. Seven other patients had more than one relative with history of POI, four of which involved their mother and sister, two their mother and maternal aunt and one their mother and grandmother. Only one of the POI patients with family history had primary amenorrhea.

No women had a fluctuating form of POI, i.e. they did not experience spontaneous resumption of their menstrual cycles and/or normalization of their FSH levels after diagnosis. Twenty two of these women had spontaneous pregnancies before the onset of POI.

Hormonal Evaluation

The mean FSH serum level was 85,2 ± 38,5 mUI/mL and the mean LH level was 43,2 ± 28,2 mUI/mL. Twenty eight (68,3%) patients had E_2 serum levels <20 pg/mL and the other 31,8% vary between 21,0 and 67,9 pg/mL and the mean was 33,5 ± 15,7 pg/mL. Three of these patients had LH levels higher than their FSH levels.

Autoimmunity

Nine (22%) women presented with personal history of one or more autoimmune diseases. Six patients had autoimmune tiroiditis with antithyreoglobuline and/or thyreoperoxidase antibodies or personal history of tiroidectomy. One patient had Beçhet's disease, one had type one diabetes mellitus and two had psoriasis, one of which had also autoimmune tiroiditis.

Pelvic Ultrasonography

Forty patients underwent transvaginal pelvic ultrasonography. Ovaries were not found in 12 patients, one of which had primary amenorrhea. Antral follicles were observed in 6 patients and 22 patients had no visible follicles in their ovaries, one of which with primary amenorrhea.

Bone Mineral Density

Osteodensitometry data was available for fourteen patients, whose median age at the time of diagnosis was 38,6 years [29-51] and the median time since the diagnosis of POI was 3,7 years. According to the World Health Organization (WHO) definition, 4 (28,6%) women had normal BMD, osteopenia in either lumbar spine or the femoral neck was found in 7 (50,0%) patients, 2 (14,3%) patients had osteopenia at both lumbar spine and femoral neck and osteoporosis was found in one (7,1%) patient.

Karyotype Abnormalities

Thirty seven patients underwent karyotype study, whose results are presented in figure 3 and table 1.

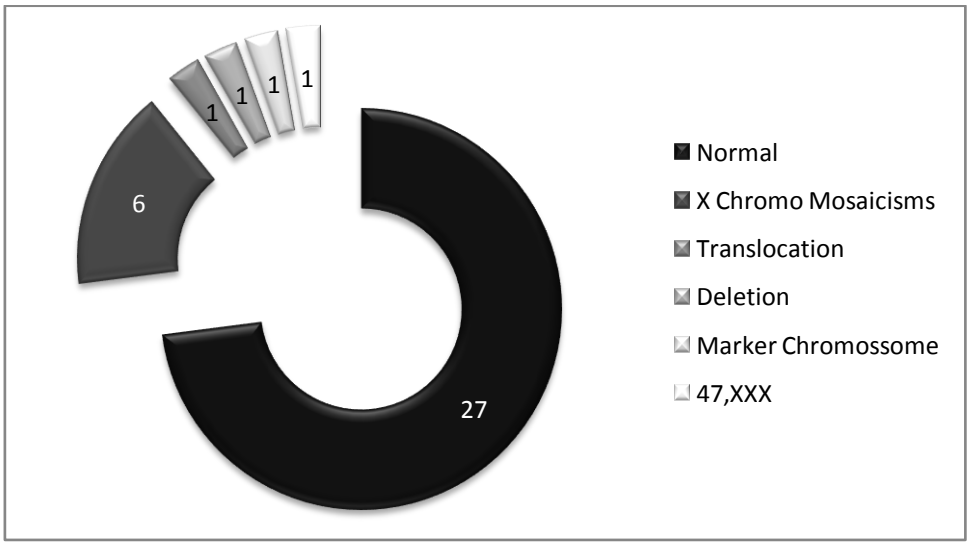

Figure 3: Distribution of karyotype abnormalities in 37 POI patients.

Karyotype Abnormalities	Number of Patients
46,X,del(q25~q26).ishdel(X)(DXYS61-)	1
mos45,X[2]/46,XX[28]	1
mos45,X[3]/46,XX[29]	1
mos45,X[2]/46,XX[48]	1
mos47,XXX[3]/45,X[1],/46,XX [26]	1
47,XX,+mar.ishder(14/22)(D14z1/D22t1+,D22S75-)	1
mos45,X[2]/46,XX[28].nucish(DXZ1x1)[4/110]	1
45,XXder(13;14)(q10;q10)	1
mos45,X[1]/47,XXX[1]/48,XXXX[1]/46,XX[47]	1
47,XXX	1

Table 1: Types of karyotype abnormalities in the 10 women with POI that had an abnormal karyotype, all with secondary amenorrhea.

FMR1 Analysis

The evaluation of the CGG number at the *FMR1* gene was performed in all patients. Five women (12,2%) had between 41 and 54 CGG repeats and two women (4,9%) had the premutation, both of which had each a child with FXS and family history of POI. The remaining women showed normal results (Figure 5).

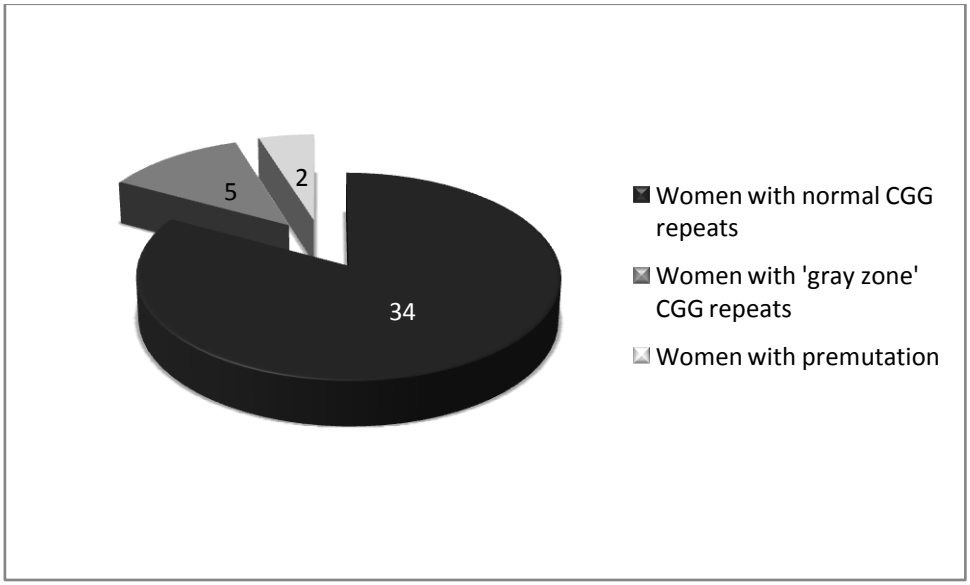

Figure 4: Distribution of CGG repeats in *FMR1* gene.

DISCUSSION

Although it has been reported in literature that POI occurs more frequently in women with primary amenorrhea than secondary amenorrhea [5], in the present study most of the women (95%) evaluated presented with secondary amenorrhea and normal puberty, probably due to the limited number of our cohort.

The present study evaluated clinical, biological, ultrasonographic and genetic data in 41 women with POI and they had a mean age of 35,5 ± 4,3 years.

From the forty patients who underwent transvaginal pelvic ultrasonography, only six (15%) presented with antral follicles, a number inferior to those in literature (30-60%) and with no apparent association between the number of follicles detected and the duration of amenorrhea [17]. Thirty four women (85%) either had no visible follicles in their ovaries or their ovaries were not found. It seems that in POI a molecular defect of genes involved in oogenesis, genes involved in the formation of the ovarian reserve, genes involved in apoptosis or genes involved in follicular growth, may be the cause of this absence of follicles or the blockage in their maturation [2].

In respect to the hormonal involvement in women with POI, we observed in this study that the mean FSH serum level was higher than normal (85,2 ± 38,5 mUI/mL) as was the mean of LH level (43,2 ± 28,2 mUI/mL). A FSH level within the menopausal range does not mean ovarian follicle depletion in POI and approximately 50% of the women experience intermittent and unpredictable ovarian function that can last for many years, meaning that these women can have a 5-10% chance of conceiving at some time after diagnosis [17].

Regarding the osteodensometry in this study group, of the fourteen women evaluated only 28,4% had a normal BMD, according to WHO definition. 71,6% of the patients experienced alterations in the BMD, suggesting that women with POI should require long-term hormone replacement therapy to prevent consequences of estrogens deficiency such as

osteoporosis, fact that was associated with hypoestrogenism for 68,3% of our patients had E_2 serum levels <20 pg/mL.

The aetiology of POI is varied [6,7] and in the present study it was possible to identify this aetiology in 19 (46,3%) patients: nine (22%) because of an autoimmune disease (5 with autoimmune tiroiditis, 1 with autoimmune tiroiditis and psoriasis and 3 with Beçhet's disease, type one diabetes mellitus or psoriasis); eight (20%) because of karyotype abnormalities (6 with X chromosome mosaicisms, one with a deletion and one with 47,XXX); and two (4,9%) because they presented the *FMR1* premutation.

The main cause of POI described in literature is associated with idiopathic and spontaneous ovarian failure associated with a normal karyotype, which is observed in our study.

We found an autoimmune cause in 22% of the cases, which is within the 10-30% of the cases in women with POI previously described [5,18], although lack of screening for other autoimmune causes, such as Addison's disease (10%) by anti-adrenal antibodies or screening for autoantibodies against ovarian tissue [2,4] constitutes a limitation to the present study.

Although primary amenorrhea is more frequently associated with an abnormal karyotype [2], in the present study the patients with karyotype abnormalities (24,4%) had secondary amenorrhea, highlighting the importance the X chromosome has in ovarian function and POI aetiology [6]. Of the ten women with karyotype abnormalities, eight could be associated with POI and all of them had the X chromosome envolved. Six women were associated with X chromosome mosaicisms, which may accelerate follicular atresia by aberrant chromosome pairing during meiosis, by deficiency or overexpression of genes that influence the oocyte quality and by defects in the meiosis-mitosis process, resulting in aberrant meiosis, gonadal damage and oocyte atresia [6]. The two main critical regions for deletions in chromosome X are located at Xq13.3-q22 and Xq26-q28 [19] and we had one

case with a deletion in Xq25-q26, which is close to the second critical region. A triple X (47,XXX), which could lead to meiotic alterations and thus ovarian failure, is still associated with uncertain significance as the incidence of 47,XXX and POI are similar, meaning that coincidental occurrence is not unexpected [20]. Although one can always argue that a 47,XXX karyotype in peripheral blood does not exclude a low level mosaic with a variable distribution in the gonads. The marker chromosome (47,XX+mar.ishder(14/22)) and the robertsonian t(13;14) also found in the present study are most probably only coincidental findings with no association with POI.

The 20% of the cytogenetic alterations observed in this study, supports that karyotypes must be obtained from all patients with POI in order to determine a possible cause and its transmission for future generations.

A familial form of POI and recent epidemiological evidence showing an association between age at menopause of mothers and daughters have been described. Also, a vertical transmission through either the maternal or paternal relatives, suggesting an autosomal or X-linked, sexlimited dominant pattern of inheritance in POI has been suggested [5,21]. In the present study, 39% of the patients had a family history of POI, 62,5% of which involved their mother and/or sister, fact that corroborates that there is a vertical transmission, even though incomplete penetrance may interfere with inheritance pattern assessments [21].

The prevalence of *FMR1* premutation in our cohort was 4,9 % (2 women), in the range of other studies (0,8-7,5%) with sporadic presentations [2,4]. Also, the prevalence of POI in women with *FMR1* premutation was described to be 13-26% [8,10,11]. Moreover, the existence of a significant association between premutation *FMR1* carriers and POI is shown by the analyses of women carrying the premutated allele and by the screening of women with POI [5,22], even though the molecular mechanism underlying the link between *FMR1* premutation and POI is at present unknown [22]. A study with strong experimental evidence

shows that not only the CGG repeats amplification might cause a change of the cellular *FMR1* expression but other genomic alterations are obviously regulating the *FMR1* transcript level and therefore the presence of POI [8].

The two premutation carriers in the present study had each a child with FXS, meaning that premutated alleles are susceptible to expansion when passed from a carrier to offspring [12,22]. Therefore, premutation of the *FMR1* gene should be evaluated in cases of familial POI in order to enable genetic counselling, where women should be informed they are at risk of having a child with FXS and thus limiting the transmission of FXS to future generations. The genetic counselling will identify also female relatives of the same risk. Furthermore, in these families it is important to offer prenatal diagnosis and the possibility of medical abortion when the mutation in the fetus is identified.

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ANNEX

The *International Journal of Gynecology and Obstetrics* (IJGO)—which is the official publication of the International Federation of Gynecology and Obstetrics (FIGO)—publishes articles on all aspects of basic and clinical research in obstetrics/gynecology and related subjects, with emphasis on matters of worldwide interest. See http://www.ijgo.org for the IJGO Statement of Purpose.

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(2) that all authors have reviewed the final version of the manuscript and approve it for publication;
(3) that neither this manuscript nor one with substantially similar content by the authors has been published elsewhere or is being considered for publication elsewhere;
(4) that it has been submitted with the full knowledge and approval of the institution or organization given as the affiliation of the author(s); and
(5) that they have informed the editor in a cover letter and in the manuscript itself of any conflicts of interest.

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3. COVER LETTER

All submissions must be accompanied by a cover letter. The letter, which should be addressed to the Editor-in-Chief (Dr Timothy Johnson), should briefly describe the study/paper and state the word count, in addition to any conflicts of interest for any of the authors.

If copyright permission is required to reproduce any material in an article, include confirmation in the cover letter that such permission has been obtained from the copyright holder (see section 5.10.2).

If any writing assistance—other than copy editing—was provided, details (including information about any funding for such assistance) must be given in the cover letter.

4. RANDOMIZED CONTROLLED TRIALS (RCTS) AND SYSTEMATIC REVIEWS

4.1. EQUATOR Network

The EQUATOR Network website (http://www.equator-network.org) explains what reporting guidelines are and why they are needed. It contains links to the checklists described below and provides useful guidance for authors and editors.

4.2. RCTs

Submission of RCTs must include reference to ethics approval (or explanation of why ethics approval was not received). Authors must consult the CONSORT statement and checklist (http://www.consort-statement.org/consort-statement/) and submit a CONSORT flow chart as an editable figure in Word/PowerPoint format.

Information regarding power calculations must be included for RCTs (see section 5.4.1).

4.2.1. RCT registration

All RCTs must be registered in a **public trials registry.**

Trials that began before July 1, 2005 The IJGO will consider "retrospective registration" of trials that began before July 1, 2005 (retrospective meaning registration occurs after patient enrollment began).

Trials that began after July 1, 2005 The IJGO will consider trials beginning on or after July 1, 2005, only if registration occurred before the first patient was enrolled (prospective registration).

The clinical trials registration information should be included at the end of the abstract.

4.3. Systematic reviews

Reviews based on the following recommended guidelines and checklists will be given preference. Systematic reviews and meta-analyses should follow the PRISMA guidelines (http://www.prisma-statement.org). Meta-analyses of observational studies should follow the MOOSE guidelines (http://www.consort-statement.org/mod_product/uploads/MOOSE Statement 2000.pdf).

5. LAYOUT OF MANUSCRIPTS

Manuscript text should be in English (US spelling), double-spaced, font size 12, in Arial font.

5.1. First page

The first page of the manuscript should contain the following: (1) title; (2) full names of authors (6 maximum, although listing more authors may be considered on an individual basis if authorship requirements have been met and a request has been included in the cover letter); (3) affiliations of authors (i.e. department, section or unit of an institution, hospital or organization, city, and country where it is located; please note that street name/numbers are not required); (4) full contact details (postal address, phone/fax numbers, e-mail address) of the corresponding author; (5) a list of up to 8 keywords for indexing and retrieval; (6) synopsis (no longer than 25 words, stating the primary conclusion of the paper).

Footnotes linking author names to affiliations should be listed as ^{a,b,c} etc., rather than *,†,‡ or ^{1,2,3} etc.

The first page should also list the type of article: Clinical Article; Brief Communication; or Review Article.

5.2. Abstract

5.2.1. Clinical articles

A structured abstract not exceeding **200 words** is required for all full-length clinical articles. It should contain all and only the following headings: **Objective**; **Methods**; **Results**; and **Conclusion.**

The Objective reflects the purpose of the study: that is, the hypothesis that is being tested. The Methods should include the setting for the study, the participants (number and type), the treatment or intervention, and the type of statistical analysis. The Results include the outcome of the study and statistical significance, if appropriate. The Conclusion states the significance of the results.

5.2.2. Systematic reviews

A structured abstract not exceeding **200 words** is required for systematic review articles (**Background**; **Objectives**; **Search strategy**; **Selection criteria**; **Data collection and analysis**; **Main results**; and **Conclusions**).

5.2.3. Brief communications

Brief communications should not include an abstract.

5.3. Main text

In full-length articles, subject matter should be organized under the following headings, with no subheadings: **Introduction**; **Materials and methods**; **Results**; **Discussion**; **Acknowledgments**; **Conflict of interest**; and **References.** Footnotes should be avoided and their contents incorporated into the text.

Brief communications should not have any headings separating the text.

5.3.1. Clinical articles

The main text of clinical articles should not exceed **2500 words**, excluding the first-page information, abstract (**no more than 200 words**), acknowledgments, conflict of interest, references (**no more than 25**), figure legends, and tables and figures. Please include the word count in the cover letter and on the first page of the manuscript.

5.3.2. Systematic reviews

Systematic reviews should adhere to PRISMA or MOOSE guidelines, with no more than **3000–3500 words** in the main text and **40 references.** Please include the word count in the cover letter and on the first page of the manuscript.

5.3.3. Brief communications

Brief communications should be no more than **400 words**, excluding the first-page information, synopsis, keywords, acknowledgments, conflict of interest, references, figure legends, and tables and figures. There should be no more than **4 references** and no more than 1 table or 1 figure. Please include the word count in the cover letter and on the first page of the manuscript.

5.4. Power calculations, statistics, and reporting of numbers

5.4.1. Power calculations

Where appropriate (e.g. for RCTs), power calculations should be performed as part of the study design, and a statement providing the power of the study should be included in the Materials and methods.

5.4.2. Statistics

The statistical tests used and the significance level set should be listed in the methods for all studies that employed statistical analysis. Information regarding the statistical software programs used should be included in the methods: for example, "SPSS (IBM, Armonk, NY, USA)." This information should not be included in the reference list.

P values should be provided where calculated. The largest *P* value that should be expressed is $P>0.99$. The smallest *P* value that should be expressed is $P<0.001$.

5.4.3. Reporting of numbers

Authors are urged to ensure that all reported numbers are accurate and listed consistently throughout the manuscript, tables, and figures.

5.5. Ethics approval and informed consent

Studies of patients, patient records, or volunteers require Ethics Committee approval and informed consent.

5.5.1. Ethics approval

Include a statement in the methods that the research protocol was approved by the relevant Institutional Review Board or Ethics Committee before the study began; if such

approval was not needed/obtained, include an explanation. Authors must provide copies of the appropriate documentation if requested.

5.5.2. Informed consent

Include confirmation in the methods that all human participants gave written informed consent before the study began; if consent was not needed/obtained, include an explanation. Authors must provide copies of the appropriate documentation if requested.

5.6. Acknowledgments

Include financial acknowledgments only.

5.7. Conflict of interest

A conflict-of-interest statement must be included in the cover letter and before the reference list in the manuscript. It should list any relationships (for any author) that may be deemed to influence the objectivity of the paper, or state that no such relationships exist.

5.8. References

The number of references should not exceed **25 for clinical articles**, **40 for review articles**, and **4 for brief communications**; in general, they should be limited to the last decade. They must be numbered and listed as they are cited in the article, using Index Medicus abbreviations for journal titles. List all authors, but if there are more than 6 list the first 6 plus "et al." Include the volume and issue numbers.

[1] Vellacott ID, Cooke EJ, James CE. Nausea and vomiting in early pregnancy. Int J Gynecol Obstet 1988;27(1):57–9. **[Journal]**

[2] Speroff L, Glass BH, Kase NG. Clinical Gynecologic Endocrinology and Infertility. Baltimore: Williams and Wilkins; 1982. **[Book]**

[3] Disaia PJ, Creasman WT. Invasive Cancer of the Vulva. In: Disaia PJ, Creasman WT, eds. Clinical Gynecologic Oncology. St Louis: C.V. Mosby; 1984:214–9. **[Chapter in book]**

[4] World Health Organization. WHO Recommended Surveillance Standards. Second Edition. http://www.who.int/csr/resources/publications/surveillance/whocdscsrisr992.pdf. Published 1999. Accessed January 15, 2012. **[Online]**

Text references should be indicated by **Arabic numerals in square brackets on the line (not superscript)**: for example, [1–4] and [1,5,11,17]. To avoid any delays in the editing process, authors must make every effort to ensure that each reference is correct and complete. Incomplete references will be returned to the principal author for completion before the manuscript is edited.

All references must be in English. Citation information of those originally in other languages must be translated into English in the reference list. The IJGO should be cited as Int J Gynecol Obstet in the reference list.

Numbered references to personal communications, unpublished data, statistical software, or manuscripts that have not been accepted for publication (i.e. "submitted" or "under consideration") must not be included. Reference to such material, if required, can be incorporated at the relevant location in the text.

If bibliographic software has been used for managing the reference list (e.g. EndNote or Reference Manager), the reference list and citations must be unlinked before submission.

5.9. Tables

Each table should be titled, numbered (with Arabic numerals), and placed on a separate page after the reference list (not embedded within the main text).

All tables must be created and submitted in editable Word format. Only standard, universally understood abbreviations should be used. Authors should prepare tabular material in an easily readable form, eliminating tables presenting information that can easily be incorporated into the text. All tables must be cited in numeric order in the main text as "Table 1" etc.

Use the Word table function (not the "enter" key, spaces, or the "tab" function) to create a separate cell for each table entry.

Footnotes to tables should be listed as ^{a,b,c} etc., rather than *,†,‡ etc.

If tables are deemed to be too large or there are too many, they may be published as online-only supplementary material (see section 5.11).

5.10. Figures and photographs

Advice on the preparation of electronic artwork can be found at the following URL: http://www.elsevier.com/artworkinstructions.

Figures and photographs should be submitted as separate figure files (not embedded within the manuscript file), preferably in TIFF or JPEG format (at least 300 dpi). CONSORT

flow charts should be created and submitted as editable Word/PowerPoint files. All figures must be cited in numeric order in the main text as "Figure 1" etc.

If labeling images, use lettering that remains clearly readable even after reduction to approximately 66%.

There are no charges for color figures.

5.10.1. Figure legends

For every figure, a titled legend must be provided in the manuscript file after the reference list; legends should be numbered consecutively in the order of their citation using Arabic numerals.

5.10.2. Figure permission

All authors wishing to use figures (or any material) that have already been published must first obtain the permission of the original author and publisher and/or copyright holders, in addition to giving precise reference to the original work. This permission must include the right to publish in electronic media. Confirmation should be included in the cover letter (the actual permission correspondence from the copyright holder does not need to be submitted).

5.10.3. Photograph/video consent

If photographs or videos of identifiable people are used, authors must obtain and submit a signed statement of informed consent from the identifiable person(s) or their next of kin. Authors should not try to conceal identity with black bars over eyes etc.

5.11. Supplementary material

Authors may submit supplementary material such as additional tables and figures, animations, presentations, and short videos. Supplementary material will be hosted online only (it will not appear in the print version).

Supplementary material will not be edited or formatted but the editors and reviewers may suggest changes. All patient/participant identification must be removed or informed consent must be obtained from the identifiable person(s) or their next of kin (see section 5.10.3).

All supplementary material should be cited in the main text of the article as "Supplementary Material S1" etc.

Supplementary material will not be accepted if the associated manuscript is rejected.

6. EDITORIAL STYLE

Papers are published in English, using US spelling. The editors reserve the right to make any necessary editorial changes.

6.1. Numerals

Arabic numerals should be used for weights, measures, percentages, and degrees of temperature. Weights and measures should be abbreviated according to the International System of Units (SI) or non-SI units mentioned in the SI: kg, g, mg, µg, mmol, µmol; m, cm, mm, µm, nm; A; cm^2; mL, µL; M, mM, µM, nM; N; d, h, min, s, ms, µs. Provide percentages after numerals throughout.

6.2. Drugs

Give **generic names** of all pharmaceutical preparations and, where appropriate, include (in parentheses, following) the trade name and manufacturer's name and address. Review drug names and dosages with care. **The author is responsible for all recommended dosages.**

6.3. Manufacturer information

Give the manufacturer's name and address (in parentheses) following the name of any instruments or equipment cited by brand name. Do not include the trademark or registered trademark symbol.

6.4. Plagiarism

Plagiarism entails the "use or close imitation of the language and thoughts of another author and the representation of them as one's own original work." Verbatim copying of sentences, even if a citation is provided (unless the sentence appears in quotation marks), is considered to be plagiarism. **Papers are checked for plagiarism (including self-plagiarism); if plagiarism is detected, action will be taken following the Committee on Publication Ethics guidelines.**

6.5. Language editing

Authors whose first language is not English are encouraged to have their manuscripts reviewed by a native English speaker or a professional editing service (e.g.

http://webshop.elsevier.com/languageservices) before submission. It is important for all submissions to be clear and understandable for the editors and reviewers.

7. PROOFS AND REPRINTS

Printer's proofs and a reprint order form will be e-mailed to the principal author by the publisher after article acceptance. They must be returned to the publisher within 48 hours of receipt. The author is responsible for detecting typesetting errors, and no major changes in—or additions to—the edited manuscript will be allowed at this stage. No free reprints will be supplied. Authors should supply a complete address for reprint requests and should return the form even if no reprints are ordered.

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