**Revisor B**

“Resumo” and Abstract

1.1.    “Nem as anomalias cromossómicas nem a FMR1-PM influenciaram a
idade de menopausa ou os níveis de FSH aquando do diagnóstico.”

1.1.1.The sentence should be clearer. It seems that chromosomal
abnormalities and FMR1 PM found are similar to that found in normal
population, and that they are not associated with POI.

We appreciate the comment. We corrected the sentence in the manuscript: “Neither chromosome abnormalities nor *FMR1* premutation influenced age at menopause or FSH levels at diagnosis in premature ovarian insufficiency patients.”

“Nem as anomalias cromossómicas nem a pré-mutação *FMR1* influenciaram a idade de menopausa ou os níveis de FSH aquando do diagnóstico na população com insuficiência ovárica prematura.”

• Introduction

2.1.    …of POI 4–6. hromosome….

2.1.1.Please correct to “…of POI 4–6. Chromosome…”

The mistake was corrected in the manuscript.

2.2.    “. A non linear relation between the number of CGG repeats and
the development of POI has been reported, although the number of repeats
associated with the highest risk is still a matter of debate 12–14”

2.2.1.Please make this sentece more clear to the reader.

The authors would like to thank the suggestion. The sentence was corrected: “A relation between the number of CGG repeats and the development of POI has been reported, although the number of repeats associated with the highest risk is still a matter of debate”

2.3.    “These figures highlight the importance of the genetic
characterization of these patients, both at the chromosomal and molecular
level.”

[2.3.1.In](http://2.3.1.in/%22%20%5Ct%20%22_blank) the introduction much is said about the relation between FMR1
mutations and POI  (and well), but litle about chromossoal abnormalities.
Since they were evaluated in the research and it is said that the patientes
shoud be evaluated “ at the chromosomal and molecular level”, I sugest
more should be said in this section about the relation between chromossomal
abnormalities and POI.

The authors totally agree with the reviewer. We added the following paragraph in the introduction: “Numerous karyotypic abnormalities have been reported, ranging from X chromosome deletions, X-autosome translocations or X-isochromosomes to numerical defects 4. X-monosomy, both with and without mosaicism, has been associated with an accelerated follicular atresia 4. 47,XXX patients are also at risk for POI, with a reported prevalence varying between 1.5% and 3.8%. The exact mechanism is not clear but an association with autoimmune diseases or a meiotic disturbance caused by an extra X chromosome have been proposed 4,7,9. In 1973, Sarto et al defined a X chromosome critical region from Xq13-Xq21 to Xq23-q27 10. The implication of this region in translocations or deletions was associated with POI. Multiple studies have corroborated this finding 4.

• Methods

3.1.    Something should me said, on the reason why not all patients were
completely evaluated. 94 patients in the study, 85 perform karyotype, 90
FMR1 analysis….

This information was added in the results:

“Due to missing data, the karyotype was analysed in 85 patients (Table 2).“

“Due to missing data, *FMR1* analysis was performed in 90 patients (Table 3).”

• Results

4.1.    Please consider changing from “…nulliparity rate was 37.2%
(n=32) and 19.8% (n=17) patients had a history of…” to “…nulliparity
rate was 37.2% (n=32) and 19.8% (n=17) of the patients had a history
of…”

We appreciate the comment. The correction was added in the manuscript.

4.2.    “The most frequent CGG number of repeats was 30 (n=53),
followed by 31 (n=19) and 29 (n=18)”

[4.2.1.Is](http://4.2.1.is/%22%20%5Ct%20%22_blank) this relevant to the evaluation? Is this outside what is observed
in the normal population?

Since the aim of our work was to provide the cytogenetic and FMR1 premutation characterization of a Portuguese population with POI, we believe these population-based differences are relevant for the scientific community. We reformulated the explanation in the discussion section in order to make it more clear: “The most frequent number of CGG repeats has been reported as 32 4, while in our sample the most frequent allele was 30. This probably reflects population related variations, which account for the importance of the genetic characterization of these patients on a population based level.”

4.3.    “… premutated alleles [(30,60) and (35,58)].”

4.3.1.Please clarify the used nomenclature

The premutated alleles included allelles ranging from 55 to 200 repeats. “Both patients with a family history of X Fragile Syndrome carried premutated alleles [(30,60) and (35,58)]. “

• Discussion

5.1.    In an Italian study, Baronchelli et al also reported a mean age
at menopause of 34 years 24. However, in this study the authors considered
patients with menopause before the age of 45 years.

5.1.1.If the second sentence refers to the Italian study, it should me more
clearly.

We appreciated the comment and added the reference after the second sentence: “In an Italian study, Baronchelli et al also reported a mean age at menopause of 34 years 26. However, in this study the authors considered patients with menopause before the age of 45 years 26”

5.2.    The prevalence of primary amenorrhea in our population was 4.3%,
which is lower than in previously analysed populations (13.2%-51.0%)

5.2.1.Something should be said about this observation.

We added the following sentence in the discussion: “We hypothesized that the fact that our department assists predominantly adult patients might have contributed to this bias.”

5.3.    “…in patients with primary amenorrhea than in patients with
secondary amenorrhea 8,26,28, in our study the figures were similar (6.7% vs
4.8%). Most certainly, the small size of the primary amenorrhea subgroup
(n=4).”

5.3.1.If only four patients, from the cohort, had primary amenorrhea, how
6.7% had chromosomal abnormalities? Please recalculate the percentage and review the sentence.

We appreciate the comment and apologize for the incorrection. In fact, as stated in the results section, none of the patients with primary amenorrhea presented with karyotypic abnormalities. This was corrected in the text: “Despite the fact that previous studies found a higher prevalence of chromosome abnormalities in patients with primary amenorrhea than in patients with secondary amenorrhea 8,28,30, in our study none of the cases with primary amenorrhea presented karyotypic abnormalities. Most certainly, the small size of the primary amenorrhea subgroup (n=4) was underpowered to provide these differences.”

5.4.    “…involving almost 135.000 patients which also---“

5.4.1.Please characterize the patients from the cited study. Were they POI
patients? Please reformulate the sentence to make it more clear.

The sentence was reformulated: “This is in line with a previous study involving almost 135.000 women from an unselected pan-ethnic cohort, which also reported a lower incidence of *FMR1*-PM in Asian patients 41.”

5.5.    “The most frequent number of CGG repeats has been reported as
32 4. In our sample, the most frequent allele was 30, which probably
reflects population related variations.”

[5.5.1.Is](http://5.5.1.is/%22%20%5Ct%20%22_blank) this relevant for the propose of the study? If you consider yes,
something more must be said about it.

We would like to thank for the comment. In fact, we believe this difference reflects population-based differences. Since the purpose of the study was to provide the genetic characterization of a Portuguese POI population, we believe this information is important. We reformulated the sentence: “The most frequent number of CGG repeats has been reported as 32 4, while in our sample the most frequent allele was 30. This probably reflects population related variations, which account for the importance of the genetic characterization of these patients on a population based level.”

**Revisor D**

* Resumo

As anomalias cromossómicas contribuem para 10% dos casos de insuficiência ovárica prematura (POI) estando maioritariamente associadas ao cromossoma X. A pré-mutação do gene*Fragile Mental* *Retardation 1 (FMR1)* tem uma prevalência estimada de 1-7% nos casos esporádicos e até 13% nos casos familiares.

É UM FACTO??? FONTE???

Agradecemos o comentário. No entanto, segundo as regras da revista Acta Médica Portuguesa, “Os resumos não podem remeter para o texto, não podendo conter citações nem referências a figuras”. Assim, as referências relativas à informação que consta na introdução do resumo encontram-se na introdução manuscrito.

O nosso objectivo foi descrever as características clínicas e a análise citogenética e do gene FMR1 numa população Portuguesa com POI. ONDE ESTÁ DEFINIDO POI???

A definição de POI encontrava-se no primeiro período do resumo. No entanto, seguindo as regras da revista, foram retiradas todas as abreviaturas do resumo.

Os *outcomes* É MESMO ISTO??? principais foram a prevalência de anomalias cromossómicas e da pré-mutação *FMR1* (*FMR1*-PM) numa população Portuguesa com POI.

Agradecemos os comentários. Substituímos “*outcomes*” por “desfechos”: “Os desfechos principais foram a prevalência de anomalias cromossómicas e da pré-mutação *FMR1* numa população Portuguesa com insuficiência ovárica prematura.”

Foram incluídas 94 doentes, com uma mediana de idade de menopausa de 36 anos. A prevalência de anomalias cromossómicas foi 16.5% e a maioria estavam relacionadas com o cromossoma X (78.6%).

PARA ESTES RESULTADOS NÃO FORAM USADOS 94 DOENTES!!!

A prevalência de *FMR1*-PM foi de 6.7%. AGORA ESTÃO A USAR 90 PACIENTES!!!

Agradecemos a correcção e modificámos o texto: “Foram incluídas 94 doentes, com uma mediana de idade de menopausa de 36 anos. A prevalência de anomalias cromossómicas foi 16.5% (14/85) e a maioria estavam relacionadas com o cromossoma X (78.6%, n=11). A prevalência da pré-mutação *FMR1* foi de 6.7% (6/90).”

* Introduction

Premature ovarian insufficiency (POI) is defined as the loss of ovarian function before the age of 40 and affects approximately 1% of women 1. Clinically, it may present as primary or secondary amenorrhea or as olygomenorrhea 1.

Reformulámos a frase: “Clinically, patients may present with primary or secondary amenorrhea, or with olygomenorrhea 1”

hromosome abnormalities are known to be present in 10-13% of patients with POI, mostly associated with X chromosome 4,7,8

Corrigimos o erro: “Chromosome abnormalities are known to be present in 10-13% of patients with POI, mostly associated with X chromosome 4,7,8

* Methods

*Statistical analysis*

Statistical analysis was performed using SPSS Statistics, Version 23.0 (IBM Corp., Armonk, NY, USA). Categorical variables were compared using the chi-square test NUNCA DEVE TER SIDO USADO UMA VEZ QUE AS AMOSTRAS SÃO DE PEQUENA DIMENSÃO, PELO QUE SE DEVE TIRAR ESTA REFERÊNCIA or the Fisher exact test according to the Cochrane rules. The normality of continuous variables was assessed with the Kolmogorov-Smirnov test. - >DEVE SER RETIRADA ESTA REFERÊNCIA POR SER INUTIL. Quantitative non-normal variables were expressed as median (interquartile range) and the non-parametric Mann-Whitney U test was used for DISTRIBUTION comparisons. All tests were 2 tailed, and p < .05 was considered statistically significant.

Agradecemos a correcção e reformulámos: “Statistical analysis was performed using SPSS Statistics, Version 23.0 (IBM Corp., Armonk, NY, USA). Categorical variables were compared using the Fisher’s exact test according to the Cochrane rules. Quantitative non-normal variables were expressed as median (interquartile range) and the non-parametric Mann-Whitney U test was used for distribution comparisons. All tests were 2 tailed, and p < .05 was considered statistically significant.”

* Results

*3.1 Clinical characteristics*

A total of 94 patients enrolled the study. Patients’ gynecological and family history is shown in Table 1. Median age at menopause was 36.0 (6.0) years). The majority of patients reported secondary amenorrhea (95.7%, n=90). Overall, the nulliparity rate was 37.2% (n=32) and 19.8% (n=17) patients had a history of previous spontaneous miscarriage THIS PERCENTAGES ARE CALCULATED WITH 86 PATIENTS The prevalence of primary amenorrhea was similar between familial (4.3%, n=1 IN 23 PATIENTS) and sporadic (4.5%, n=3 IN 67 PATIENTS) WHY 90??? YOU ARE TALKING ABOUT 90 OR 94 PATIENTS??? POI patients (p=0.731 WHAT TEST?? WHAT IS THE VALUE OF THE TEST STATISTIC, AND DEGREES OF FREEDOM???), and between patients with (0%) and without (4.5%, n=4) a family history of Fragile X Syndrome (p=0.913). WHAT TEST?? WHAT IS THE VALUE OF THE TEST STATISTIC???),

Agradecemos o comentário extremamente construtivo e reformulámos o texto de forma a permitir uma melhor compreensão. Para além disso:

* Corrigimos o denominador dos antecedentes obstétricos, uma vez que verificámos que as 4 doentes com amenorreia primária tinham sido inadvertidamente classificadas como missing nas variáveis relativas à história obstétrica. Assim, conforme exposto no texto corrigido, a história obstétrica era desconhecida em apenas 4 pacientes pelo que o denominador é 90.
* Concordamos com a questão levantada pelo revisor sobre o valor dos testes comparativos utilizados em grupos de pequenas dimensões como os que se referem à prevalência de amenorreia primária em doentes com história familiar de IOP e Síndrome do X Frágil. Deste modo, substituímos o estudo comparativo pelo estudo descritivo nestes parâmetros

Assim, o novo parágrafo será:

“A total of 94 patients enrolled the study. Patients’ gynecological and family history is shown in Table 1. Median age at menopause was 36.0 (6.0) years. The majority of patients reported secondary amenorrhea (95.7%, n=90).

Obstetric history was unavailable in 4 patients. Overall, the nulliparity rate was 40.0% (36/90) and 18.9% (17/90) of the patients had a history of previous spontaneous miscarriage.

Twenty-three patients presented a family history of POI. The prevalence of primary amenorrhea was 4.3% (1/23) in familial cases and 4.2% (3/71) in sporadic POI patients.

A family history of Fragile X Syndrome was present in 2 patients. None of the cases with family history of Fragile X Syndrome presented with primary amenorrhea.

No statistically significant difference was found between the median FSH at diagnosis in patients with primary vs. secondary amenorrhea [64.9 (56.0) vs 80.0 (39.0) IU/l, p=0.392, Mann Whitney test].”

*3.2 Chromosomal abnormalities*

The karyotype was analysed in 85 patients IT INCLUDES THE 4 PATIENTS WITH PRIMARY AMENORRHEA??? IT INCLUDES ALL THE 23 PATIENTS WITH FAMILIAL CAUSES??? (Table 2). An abnormal karyotype was observed in 16.5% (n=14), of which 78.6% (n=11) involved the X chromosome. The most common abnormality was X chromosome mosaicism, which was found in 7/14 patients (50.0%). The 4 patients with primary amenorrhea presented a normal karyotype. No significant difference was found regarding age at menopause [35.5 (7.8) vs 36.0 (6.0) years, p=0.691] or FSH at diagnosis [83.0 (62.0) vs 78.1 (32.0) IU/l, p=0.415] between patients with (14 PATIENTS) or without an abnormal karyotype (71 PATIENTS). Also, no statistically significant difference was found regarding the prevalence of karyotypic abnormalities (YOU HAVE 14 IN THIS CONDITIONS, RIGHT???) between patients with (13.0%, n=3 IN 23) and without (19.4%, n=12 11??? IN 62) a family history of POI (23 PATIENTS) (p=0.239). THE VALUES ARE CORRECT???? WHAT TEST?? Exact FISHER??? WHAT IS THE VALUE OF THE TEST STATISTIC??),

As 85 pacientes incluem as 4 doentes com amenorreia primária e as 23 doentes com história familiar de POI. De facto, verificámos uma incorrecção nos números de doentes com do cariótipo em doentes com história familiar de IOP pelo que refizemos a análise estatística de forma a corrigir os mesmos.

O texto foi reformulado para uma melhor compreensão do mesmo:

“The karyotype was analysed in 85 patients (Table 2).

An abnormal karyotype was observed in 16.5% (n=14), of which 78.6% (n=11) involved the X chromosome. The most common abnormality was X chromosome mosaicism, which was found in 50.0% of our cohort (7/14). The 4 patients with primary amenorrhea presented a normal karyotype.

No statistically significant difference was found regarding age at menopause [35.5 (7.8) vs 36.0 (6.0) years, p=0.691, Mann Whitney test] or FSH at diagnosis [83.0 (62.0) vs 78.1 (32.0) IU/l, p=0.415, Mann Whitney test] between patients with (n=14) or without (n=71) an abnormal karyotype.

Also, no statistically significant difference was found regarding the prevalence of karyotypic abnormalities between the 23 patients with a family history of POI (8.7%, n=2) and those without (19.4%, n=12) (p=0.333, Fisher’s exact test).“

*3.3 FMR1 analysis*

*FMR1* analysis was performed in 90 patients (IT INCLUDES THE 4 PATIENTS WITH PRIMARY AMENORRHEA??? IT INCLUDES ALL THE 23 PATIENTS WITH FAMILIAL CAUSES??? ) and *FMR1*-PM was present in 6.7% (n=6) (Table 3). The most frequent CGG number of repeats was 30 (n=53), followed by 31 (n=19) and 29 (n=18). All patients with *FMR1*-PM presented with secondary amenorrhea. 6/86 IS THE PROPORTION OF PATIENTS WITH *FMR1*-PM IN THE SET OF PATIENTS WITH SECONDARY AMENORRHEA )

No significant difference was found between patients with and without *FMR1*-PM concerning age at menopause [38.0 (1.8) vs 36.0 (6.0) years, p=0.092] or FSH levels at diagnosis [84.7 (63.0.) vs 77.7 (40.0) IU/L, p=0.340]. There was a higher prevalence of *FMR1*-PM in patients with a family history of POI (3 patients in 23????) but this difference was not statistically significant (13.0% vs 4.5% 3 patients in 67???, p=0.176) WHAT TEST?? Exact FISHER??? WHAT IS THE VALUE OF THE TEST STATISTIC?. Both patients with a family history of X Fragile Syndrome carried premutated alleles [(30,60) and (35,58)].

Reformulámos o texto acrescentando a referência às 4 doentes com amenorreia primária e às 23 doentes com história familiar de IOP, bem como acrescentando os denominadores para uma melhor compreensão dos resultados:

*“FMR1* analysis was performed in 90 patients and *FMR1*-PM was present in 6.7% (n=6) (Table 3). The most frequent CGG number of repeats was 30 (n=53), followed by 31 (n=19) and 29 (n=18).

None of the 4 patients with primary amenorrhea presented the *FMR1*-PM.

No significant difference was found between patients with and without *FMR1*-PM concerning age at menopause [38.0 (1.8) vs 36.0 (6.0) years, p=0.092, Mann Whitney test] or FSH levels at diagnosis [84.7 (63.0.) vs 77.7 (40.0) IU/L, p=0.340, Mann Whitney test].

There was a higher prevalence of *FMR1*-PM in patients with a family history of POI but this difference was not statistically significant [13.0% (3/23), vs 4.5% (3/67), p=0.176, Fisher’s exact test).

Both patients with a family history of X Fragile Syndrome carried premutated alleles [(30,60) and (35,58)].”

* Discussion

Despite the controversy regarding race/ethnicity *per se* as a factor that influences age at menopause, a higher social class, the prolonged used of oral contraceptives and a higher baseline weight seem to be associated with a higher age at natural menopause. Although no epidemiological studies have been performed in POI populations, we hypothesize that these factors may also contribute to our results. WHY???

Agradecemos o comentário. De facto, estas associações foram encontradas em vários estudos. No entanto, o mecanismo causal não se encontra totalmente esclarecido. Assim, reformulámos o texto:

“Despite the controversy regarding race/ethnicity *per se* as a factor that influences age at menopause, a higher educational level, the prolonged used of oral contraceptives and a higher baseline weight seem to be associated with a higher age at natural menopause 32,33. The exact mechanism behind these associations is not completely understood. Although no epidemiological studies have been performed in POI populations, we hypothesize that these factors may also contribute to our results.”

The prevalence of primary amenorrhea in our population was 4.3%, which is lower than in previously analysed populations (13.2%-51.0%) [7,8,25,26,28,31 IT COULD BE ALSO PRESENTED A CONFIDENCE INTERVAL BASED ON THE EXACT BINOMIAL TEST [1,1%; 10,12%] WITH 95% CONFIDENCE

Agracedemos a sugestão. Acrescentámos a informação: The prevalence of primary amenorrhea in our population was 4.3% (95%CI 1.6-11.0%), which is lower than in previously analysed populations (13.2%-51.0%) 7,8,28,29,31,34.

The rate of previous spontaneous miscarriage was 19.8%, (WHO ARE THE 8 PATIENTS NOT INCLUDED???) higher WHY??? than reported by Allen *et al* and Jansel *et al* in a POI population (5.0-13.9%), but similar to the expected rate in the general population 12,25,32 IT COULD BE ALSO PRESENTED A CONFIDENCE INTERVAL BASED ON THE EXACT BINOMIAL TEST [7,95%; 22,59%] WITH 95% CONFIDENCE

De acordo com a informação modificada anteriormente, corrigimos a taxa de aborto espontâneo, uma vez que apenas 4 doentes não tinham história obstétrica disponível. Reformulámos a frase:

“The rate of previous spontaneous miscarriage was 18.9% (95%CI 12.0-28.5%), higher than reported by Allen *et al* and Jansel *et al* in a POI population (5.0-13.9%), but similar to the expected rate in the general population 15,28,35.”

The prevalence of chromosomal abnormalities in our population was 16.5%. IT COULD BE ALSO PRESENTED A CONFIDENCE INTERVAL BASED ON THE EXACT BINOMIAL TEST [7,95%; 22,59%] WITH 95% CONFIDENCE

The prevalence of chromosomal abnormalities in our population was 16.5% (95%CI 9.9-26.1%). Most studies report a prevalence of karyotypic abnormalities varying between 9% and 14% 7,8,27,28,31,34.

The prevalence of *FMR1*-PM in our sample was 6.7%,CONFIDENCE INTERVAL BASED ON THE EXACT BINOMIAL TEST [2,33%; 13,11%] WITH 95% CONFIDENCE

similar ???? to what has been previously described in non-Asian populations 1,26.

Acrescentámos o intervalo de confiança conforme sugerido:

“The prevalence of *FMR1*-PM in our sample was 6.7% (96%CI 3.0-14.2%), similar to what has been previously described in non-Asian populations 1,29.”