Author's response to reviews

Title: Streak ovaries in association with Aromatase deficiency due to a novel CYP19A1 mutation

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Author's response to reviews:

25 November 2013

The Editor
BMC Endocrine Disorders

Dear Editor

RE: Manuscript (Case report) entitled:
A case of Aromatase deficiency due to a novel CYP19A1 mutation
Thank you for considering our revised manuscript for publication in BMC Endocrine Disorders.

We sincerely thank the reviewers for their thoughtful comments and feedback. We have revised the manuscript in accordance with the recommendations. We believe our case report is significantly enhanced following the modifications suggested by the reviewers. One of the reviewers was keen for us to present the paper in a more concise manner, and whilst we have tried to be as concise as possible several details and further discussion requested by other reviewers has resulted in an overall lengthening of the manuscript.

Please find to follow for each reviewer, each of their comments and our considered responses. We have also indicated where our amendments can be found in the revised manuscript (page and line numbers) and in most cases have reproduced the amendment directly from the manuscript for the reviewers’ convenience. We believe we have provided comprehensive responses to the concerns raised by the reviewers. We have not been able to perform in vitro functional studies of the effect of the novel mutation our patient has, on aromatase function. However, the phenotype of our patient is consistent with previous reports of aromatase deficiency in females, and moreover is also consistent with reports of aromatase deficiency in males and the aromatase
deficient murine model. Therefore we do not think the diagnosis is in doubt, and for the most part, nor did the reviewers.

We thank you again for considering this manuscript for publication. As per our previous cover letter, we have no issues with respect to the editorial policies of the journal.

Kind regards
Yours sincerely
Dr Lucia Gagliardi
Endocrinologist
on behalf of the authors

Reviewer's report
Title: Streak ovaries in association with Aromatase deficiency due to a novel CYP19A1 mutation
Version: 1 Date: 4 July 2013
Reviewer 1: Anna Lauber-Biason
Reviewer's report:
Gagliardi et al describe a patient with features of in utero hyperandrogenism and mutation in the aromatase CYP19A1 gene. The novelty of this report is the presence of ovarian dysgenesis, previously unreported in aromatase mutated patients.

Although potentially intriguing, the paper has some flaws that need to be addressed:

Major:
1. Some of the clinical characteristics of the patient do not agree with an isolated aromatase deficiency. For instance, this patient did not have adrenarche, suggesting a combined problem in adrenals and gonads. The association of in utero virilization that does not proceed later is a feature of P450 oxidoreductase (POR) deficiency. This entity is also associated with aromatase deficiency in some cases, due to the fact that POR is necessary for proper function of all microsomal P450 enzymes, including P450c21, P450c17 and P450Aro (see Flück et al, Nature 2004, Flück and Miller, Current Opinion in Pediatrics 2006). In this view, I feel it is necessary to check the progesterone levels, elevated in CYP17A1 deficiency (also in gonadectomized patients) and the sequence of POR. Oligogenic endocrinological diseases are not as rare as previously thought (see the example of hypogonadotrophic hypogonadism).

We thank the reviewer for this comment and for the suggestion. We note the concern raised that some of the clinical characteristics of the patient do not agree with an isolated aromatase deficiency – for example the patient did not have adrenarche, suggesting a combined problem in adrenals and gonads. However,
the patient did not recall having adrenarche – it is impossible for us to verify this retrospectively. Furthermore, reviewer 3 raised an important point – that we should focus the clinical presentation on our assessment, and place less emphasis on aspects of the past history in particular when some aspects we can not verify. Thus, we have removed reference to the lack of recalled adrenarche, as this is a circumstantial component of the history that we can not verify. We have however retained aspects of the past history that are indisputable (for example, ambiguous genitalia).

Thank you for advising that the association of in utero virilisation that does not proceed later is a feature of POR deficiency. We have measured the progesterone level as requested. The patient’s progesterone level was <2 nmol/L; this is not consistent with CYP17A1 deficiency. We have included the patient’s progesterone level with the other biochemical data we have presented in Table 1. We have also sequenced the coding exons (exons 2-16, including splice-sites) of P450-oxidoreductase as requested. We found the patient to have a known synonymous SNP in exon 13 (rs2228104) and a known synonymous SNP in exon 14 (rs1057870). Given that these are synonymous variations that do not cause an amino acid change we do not believe they are relevant to her phenotype. We have made reference to this data in the manuscript (Page 7, Lines 189-90).

We suggest an alternative explanation for in utero virilisation that does not proceed later:
This may reflect relatively early loss of ovarian function as a source of androgens (Page 11, Lines 287-8).

2. Sex hormones and gonadotropins levels are close to impossible to judge in gonadectomized individuals, even under estrogen replacement. I am not sure what conclusions can be drawn from these measurements.
We thank the reviewer for this comment. We wanted to include some biochemical data but felt that it was unethical to stop the oestrogen replacement to obtain untreated hormone levels; hence we have presented the biochemical data whilst the patient was taking oestrogen replacement.

3. The “homozygous” duplication can also be a “hemizygous” mutation and since the parents are not available no definitive conclusion can be made. Please discuss this point.
We thank the reviewer for raising this very important point. We have discussed the possibility that the patient may be hemizygous for the mutation, and that we can not make a definitive conclusion as her parents are unavailable as requested (Page 7, Lines 192-193). The statement is as follows:

Whilst she is most likely to be homozygous for the observed mutation, her parents were unavailable for testing and hence we are unable to exclude hemizygosity.
4. Although I realize that nowadays three-dimensional models of mutated proteins are sometimes accepted as a surrogate of functional studies, I would like to see real enzymatic functional studies or at least hard data, i.e. a Western blot, to prove that the protein is really longer.

We thank the reviewer for this comment. Unfortunately it was not possible to perform functional studies in our laboratory and we did approach a potential collaborator but were unsuccessful. We have stated the lack of functional studies as a limitation as recommended by reviewer 2 (Page 11, Lines 293-6). However, we strongly believe that the clinical phenotype leaves no doubt to the diagnosis.

We are optimistic that this will not preclude publication of this intriguing and rare case report of aromatase deficiency in a female not treated until adulthood.

We have inserted the following statement (Page 11, Lines 293-6)

A limitation of this work is that we have not performed functional studies to verify the effect of the duplication on protein function; however the clinical presentation and the correlation with cases already reported and with the ArKO mouse model is compelling for this being a loss of function mutation.

Minor

1. In the “Background” section the authors claim (page 3, lines 76-79) that the aromatization of 16OHDHEA into E1 is the major source of estrogens in pregnancy: this is not correct, since estradiol is still the major estrogen in pregnancy. This statement should be modified.

We have modified this statement as requested. The key point of the statement was that placental aromatisation of fetally-derived androgens is the major source of circulating oestrogens in human pregnancy. The statement has been rephrased as follows:

In pregnancy, placental aromatisation of 16-hydroxy-dehydroepiandrosterone sulphate, arising from foetal liver hydroxylation of dehydroepiandrosterone sulphate produced by the foetal adrenal, is the major source of circulating oestrogens; the activity of placental aromatase protects the foetus against the virilising action of foetal androgens [3]. (Page 3, Lines 75-79).

2. The major reason why aromatase deficiency was only recently described is that the dogma was that without placental estrogens there is no pregnancy. The discovery of CYP19A1 human mutants changed this dogma (together with those mentioned on page 4, lines 115-117). The statement on page 4, line 94 should be modified.

We have modified the statement as requested (Page 4, Lines 92-99) and as follows:

The recent description of aromatase deficiency, relative to other defects of steroidogenesis, is likely because oestrogens were erroneously considered
essential for human survival and necessary for blastocyst implantation, and therefore it was concluded that aromatase deficiency would be incompatible with life [13]. It was observed however that both male and female mice with disrupted oestrogen receptor genes were viable, raising speculation about the validity of the lethality hypothesis [14]. The very first case report of a female with aromatase deficiency verified that at least beginning late in the first trimester, excess androgen with low or absent oestrogen could indeed be compatible with life [8].

3. In the evaluation of ambiguous genitalia, different scales/standards are available, the most used being the Prader I-V scale. It would be good to have the Prader grading in the case presentation.

She is Prader 3. We have included this in the case presentation (Page 6, Line 156).

4. Is there anything known about the bone age of the patient?

Unfortunately, nothing is known about the bone age of the patient. By the time we first saw her she had already received several years of oestrogen treatment. We have not been able to contact the doctors in rural India who treated her at the time of her fracture, so have not been able to locate any x-rays if they were performed, and are uncertain as to the bone age at the time of the fracture.

Reviewer 2: Vincenzo Rochira
Reviewer's report:
Major Compulsory Revisions

Dear Editor,

in this manuscript, the authors describe a case of female aromatase deficiency. The data presented confirm, at least in part, what we know about this disease in women and add new very interesting data. To my opinion, however, the manuscript fails in remarking adequately the novelty represented by this case and could be largely improved by putting the results into the appropriate clinical and scientific context.

In particular, the authors should focus the discussion on these two main aspects: a) the natural history of the disease; b) the fact that this is the first case of female aromatase deficiency with both a delay in the diagnosis and treatment (see major comments for detailed suggestions). If the discussion will be rewritten bearing in mind these two aspects, the novelty of the results will be remarked, the manuscript will result improved, and some important pathophysiological aspects will come out.

This study is of great value since it confirms for the first time the absence of gender differences in the estrogen action on bone and metabolic parameters too by studying the human aromatase deficiency model in an untreated adult female.
I think that the manuscript will be suitable for publication only if the discussion and part of the results will be rewrite strictly according to the comments and the suggestions provided. If the manuscript will not shown an extensive rearrangement of the discussion focusing on the novelty, it will be not suitable for publication.

I suggest Major Compulsory Revisions.

Major Comments

1) The main difference between male and female aromatase deficiency is in the timing of diagnosis and treatment. In females the ambiguous genitalia at birth and later the primary amenhorrea prompt the diagnosis and induce the physician to treat the patient with estrogen at puberty (Rochira & Carani, Nature Reviews Endocrinology, 5:559–568, 2009). Accordingly, all previous females received estrogens at puberty and did not develop all the bone and metabolic alterations that are classically found in aromatase-deficient men (who remain usually unrecognized and untreated till the age of 30 yrs). The case here presented is anecdotal since estrogen treatment was delayed (due to several factors that are well described in the medical history) and this patient developed all the bone and metabolic features related to prolonged estrogen deprivation throughout puberty and adulthood, similarly to what happens to aromatase-deficient men. This means that estrogen deficiency (if untreated) leads to the same phenotype in both men and women. In order to parallel what happens in men and women this reference could be of help: Zirilli et al., Human models of aromatase deficiency. J Steroid Biochem Mol Biol 109:212-218, 2008. Again, this means that estrogen action are highly conserved in both sexes without gender differences.

Thank you for this comment. We have discussed this as requested and as shown below:

Page 8, Lines 219-23

A similar phenotype of tall stature, continuing linear growth into adulthood, unfused epiphyses, delayed bone age, osteoporosis and eunuchoidal skeletal proportions is well described in adult males with aromatase deficiency, which corrects with oestrogen treatment, reflecting the shared role of oestrogen in both males and females in bone maturation and mineralisation [35]. (Page 8, Lines 219-23)

Page 9, Lines 254-7

Further to our observation of metabolic syndrome in this female patient with aromatase deficiency, aromatase deficient adult men have a variable phenotype of insulin resistance, abnormal lipid profile and clinical features similar to the metabolic syndrome; with improvement in these parameters following treatment with oestrogen [18, 35, 38-40]. (Page 9, Lines 254-7)

Page 10, Lines 277-80

Whilst male and females (untreated) with CYP19A1 deficiency share a similar metabolic phenotype, there may be a gender difference in the mechanisms. High
androgen levels have been associated with peripheral insulin resistance in women, but not in men [46, 47]. The gender difference may be due to differences in the androgen-oestrogen ratio and its effects on cellular metabolism. (Page 10, Lines 277-80).

2) The patient continued to growth into adulthood and developed eunuchoid proportions as men. Please remark that this feature was related to unfused epiphyses during adulthood and continuing linear growth. Please provide adequate documentation by showing: 1) the patient’s growth chart and 2) the X-ray of the hand performed at the time of the fracture of the radius (age 25 yrs), this X-ray is needed to document open epiphyses. Add a description and a figure of the X-ray in the section ‘Case Presentation’ (page 5, lines 141-142).

We have included the following statement in the paper (Page 11, Lines 288-291):

She had eunuchoidal proportions, a result of continued linear growth in adulthood due to unfused epiphyses in the absence of oestrogen. As we have already mentioned, this is a frequent clinical observation in aromatase deficient males, who are generally not diagnosed and therefore not treated until after puberty [17]. (Page 11, Lines 288-291)

We thank the reviewer for the request of additional clinical and radiological information. Unfortunately, this information is not available, as acknowledged by the comments provided by Reviewer 3. We do not have the growth charts (and in fact are unsure whether her growth in childhood was documented since the diagnosis of aromatase deficiency, or indeed any other diagnosis, was not suspected) and the x-ray of the hand is not available. The eunuchoidal proportions of the patient that we have documented during our clinical examination, is testament to her having had ongoing linear growth and delayed epiphyseal closure, prior to oestrogen treatment.

3) Please, refer to a recent case of estrogen resistance in a woman (Quaynor SD et al. NEJM 369:164-171, 2013) that is the first one described and is very similar to the case here presented in terms of clinical feature and pathophysiological significane of the results. In particular, discuss your results in parallel with that of this case, highlighting similarities and differences.

We thank the reviewer for this comment. We have made reference to this case report and have discussed our results in parallel with that of this case, highlighting similarities and differences (Page 8, Lines 203-229, Page 9, Lines 230-233). Our discussion is reproduced here for your reference:

Several of the clinical features of our patient, in whom oestrogen treatment was delayed, were shared by the recently described first female patient with oestrogen resistance due to a novel homozygous missense mutation in a highly conserved region of exon 5 of the oestrogen receptor # (ESR1) [34]. The patient with oestrogen resistance had absent breast development, primary amenorrhoea and intermittent lower abdominal pain due to markedly enlarged multicystic ovaries [34]. She also had delayed bone age, unfused epiphyses and osteopaenia. Her oestrogen level was markedly elevated (12,848pmol/L; normal
follicular phase: 40-771pmol/L) with mildly elevated gonadotropins (follicle-stimulating hormone - 6.7-19.2mIU/mL, normal follicular phase: 1.9-12.5mIU/mL; luteinising hormone - 5.8 to 13.2mIU/mL, normal follicular phase: 2.5-10.2mIU/mL) [34]. Oestrogen resistance was demonstrated clinically by the failure of breast development following oral conjugated equine oestrogen and also oral micronized oestradiol [34]. In vitro the mutated receptor had greatly reduced activity compared with wildtype [34].

Both patients had a lack of spontaneous breast development, primary amenorrhoea and osteopaenia, highlighting the critical role of oestrogen in pubertal and bone development. The patient with oestrogen resistance had radiological evidence of delayed bone age and unfused epiphyses; in our patient x-rays were not available, however we surmise from her eunuchoidal proportions that epiphyseal fusion of long bones was delayed, consistent with the known role of oestrogen in epiphyseal maturation [34]. A similar phenotype of tall stature, continuing linear growth into adulthood, unfused epiphyses, delayed bone age, osteoporosis and eunuchoidal skeletal proportions is well described in adult males with aromatase deficiency, which corrects with oestrogen treatment, reflecting the shared role of oestrogen in both males and females in bone maturation and mineralisation [35]. These data suggest, as is already well-known in clinical practice, that long-term oestrogen deficiency may adversely affect bone health.

There are also some interesting differences between the two cases. Our patient had a phenotype of metabolic syndrome comprising central obesity, borderline hypertension, hepatic steatosis and dysglycaemia with an elevated fasting glucose and fasting insulin, consistent with insulin resistance. In contrast, the patient with oestrogen resistance had a normal BMI and no evidence of impaired glucose tolerance [34]. The absence of the metabolic phenotype in the latter case is in contrast however with the only previously reported patient with an ESR1 mutation who was male and had obesity, glucose intolerance and hyperinsulinaemia; furthermore both male and female oestrogen receptor knockout mice are obese and have insulin resistance and glucose intolerance, [36, 37].

4) In the references, please quote also a very important work written by Lin et al. JCEM 2007.

We have included this important paper in the references (reference number 15), and made reference to it in the body of the paper in the context of description of the phenotypes of patients with aromatase deficiency (Page 4, Line 107; Page 5, Line 124; Page 9, Lines 237).

5) In the introduction (page 4, lines 108-109) introduce the concept that differences in clinical features between men and women are due to different timing of treatment.

We have added the following statement (Page 4, Lines 102-104): Whilst females are usually diagnosed at birth, diagnosis in males is generally delayed until puberty – the delay in oestrogen treatment in males underscores
the phenotypic differences between the genders [17].

6) Did you performed a functional analysis of the mutated enzyme by in vitro carrier transfection? If not please list this as a limit of the study in the discussion. We did not perform a functional analysis of the mutated enzyme. We have listed this as a limitation of the study in the discussion (Page 11, Lines 293-6). However, the clinical phenotype leaves no doubt to the diagnosis.

7) The patient displays complicated obesity with hypertension. Please provide data on liver function, lipid profile, the degree of obesity (BMI, waist circumference, waist to hip ratio,) and the body fat distribution of adipose tissue at DEXA (if available). Had the patient acanthosis nigricans?

We have added data regarding her liver function and lipid profile as requested, and have inserted this data in table 1. We have presented her waist circumference as 131cm, hip circumference 115cm (waist to hip ratio 1.14) consistent with central adiposity in the description of her clinical features (Page 6, Line 168). The body fat distribution of adipose tissue at DEXA was not available. She did not have acanthosis nigricans.

How is liver morphology at US? How is fasting insulin? from a clinical point of view the patient should receive an OGTT.

Ultrasound showed the liver to be of normal size. There was no focal lesion seen. Echogenicity was generally increased. The capsular surface was smooth. The portal vein calibre was normal and portal venous flow was directed towards the liver. There was no ascites. The spleen was normal.

The reporting radiologist’s conclusion was: evidence of moderate hepatic steatosis. We have presented this in the clinical data (Page 7, Line 178).

Thank you for the suggestion of performing an oral glucose tolerance test and measuring insulin levels. The results were as follows:

<table>
<thead>
<tr>
<th>Glucose (mmol/L)</th>
<th>Insulin (mU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mins 6.1</td>
<td>57</td>
</tr>
<tr>
<td>120 mins 7.5</td>
<td>290</td>
</tr>
</tbody>
</table>

The fasting insulin is clearly elevated. The patient has impaired fasting glucose, but normal glucose tolerance. The very elevated insulin level at 2 hours, together with the elevated fasting insulin level is consistent with profound insulin resistance. We have included the results of the oral glucose tolerance testing and the fasting insulin level in table 1. We have referred to these results on Page 7, Lines 176-7. The statement is reproduced here for your reference:

Biochemical testing revealed the patient had impaired fasting glucose, dyslipidaemia and insulin resistance (Table 1). (Page 7, Lines 176-7).

8) What is the explanation for streak ovaries? Again focus better on the natural history of the disease and the prolonged estrogen deprivation in this patient as a
possible hypothesis. In the mouse model (discussion page 8, lines 213-225) of aromatase happens the same.

Thank you for this comment. We have elaborated on potential explanations for the reported streak ovaries in this patient (Page 11, Lines 298-313 and Page 12, Lines 314-25). We hypothesise that prolonged oestrogen deprivation may be one potential explanation, as there is a corresponding similar phenotype in the aromatase knockout mouse. Our discussion is shown below for your reference.

Unexpectedly, our patient was reported to have had streak ovaries excised. We caution that the histological slides were unavailable for us to verify the pathology report especially since streak ovaries have not been previously described in association with aromatase deficiency. Polycystic ovaries have been described, consistent with the increased androgenisation in tissues [18-22]. Regression of ovarian cysts with oestrogen treatment has been described in several cases [19, 20, 22, 49]. Other case reports however have described normal ovarian morphology, even in the absence of prior oestrogen treatment [10, 11]. Thus, the case reports to date suggest that there is no consistent ovarian phenotype in aromatase deficiency and that the phenotype may be modulated by treatment. Hence, in our patient, the ovarian histology may be a result of oestrogen treatment.

Alternatively, extrapolating from observations in the ArKO mouse model, we speculate that the streak ovaries may be an inherent manifestation of CYP19A1 deficiency [41, 42]. Follicular development in ArKO is abnormal in an age dependent manner, with an early block in follicular development at the antral stage with absent corpora lutea, then haemorrhagic cysts and later, absent secondary and antral follicles and atresia of primary follicles with increased collagen deposition [41, 42]. Hence, whilst not previously reported, it is possible that the longer life span of the human may permit more complete follicular atresia and collagen deposition mimicking the classical streak ovaries seen in Turner’s syndrome. Streak ovaries may not have been previously described in human CYP19A1 deficiency because direct visualization of the ovaries with laparoscopy would be infrequently performed and some of the cases reported may have been too young to develop this manifestation. In addition, some of the cases have had CYP19A1 mutations that allowed some residual aromatase function and may have a less severe ovarian atretic phenotype.

Another possibility is that our case may harbour another cause of the streak ovaries such as XO mosaicism localised to the ovaries, although the normal XX chromosomal composition in both lymphocytes and in fibroblasts makes this less likely. Streak ovaries are not clearly associated with any other disorder but Turner’s syndrome. In view of the small numbers of CYP19A1 cases reported this manifestation should be noted particularly since this finding may be relevant to ovarian developmental biology.

Did you measure antimullerian hormone?

Thank you for this suggestion. We have measured antimullerian hormone as requested. The result was undetectable (<3pmol/L) which is below the 25th
percentile for her age and is consistent with low ovarian reserve, which is as expected as she has had bilateral oophorectomy. We have included this in Table 1.

9) Please discuss in a more extensive fashion abdominal obesity and metabolic alterations.

We have provided more detail regarding the patient’s anthropometric measurements and metabolic alterations (Page 6, Line 168 and Table 1; Page 7, Lines 175-8).

We have also discussed in detail central obesity and metabolic perturbations in aromatase deficient men (Page 9, Lines 231-234), aromatase deficient women (Page 9, Lines 236-254) and also in the aromatase knockout mouse model (Page 10, Lines 256-267). We have reproduced the discussion for your reference below:

In aromatase deficient females, the relationship between carbohydrate and lipid metabolism and oestrogen deficiency is unclear. A 14 year old female with aromatase deficiency had normalisation of mild dyslipidaemia with oestrogen treatment [15]. However, another aromatase deficient female, aged nine, had early features of puberty and severe insulin resistance and glucose intolerance which progressed to overt diabetes despite treatment with oestrogen, and, subsequently, metformin [26]. Androgen levels were essentially unchanged during oestrogen treatment. Treatment with a gonadotropin releasing hormone analogue, whilst causing a profound reduction in serum androgens, was associated with only a slight improvement in glucose tolerance, but persistent insulin resistance [26]. Thus the insulin resistance could not be attributed to high androgen levels. It was postulated that exposure of the female to high levels of androgens or lack of oestrogens in utero might have altered foetal programming of insulin sensitivity. This patient’s ethnicity was South American and there was a family history of type 2 diabetes in her paternal grandparents, but none of obesity. Her growth charts revealed an increase in weight from near the 50th percentile to the 90th percentile over approximately the two years prior to the observation of elevated fasting insulin and insulin resistance, and prior to oestrogen replacement [26]. Thereafter, and whilst on oestrogen replacement, her weight increased to above the 90th, and subsequently, above the 97th percentile [26]. Thus, this patient’s ethnicity, a genetic predisposition and coincident obesity may have also been contributors to her profound insulin resistance and eventual development of overt type 2 diabetes mellitus.

Further to our observation of metabolic syndrome in this female patient with aromatase deficiency, aromatase deficient adult men have a variable phenotype of insulin resistance, abnormal lipid profile and clinical features similar to the metabolic syndrome; with improvement in these parameters following treatment with oestrogen [18, 35, 38-40]. The development of central obesity in postmenopausal women is also considered secondary to oestrogen deficiency. It is unknown, and we believe, unethical to explore, whether and by how much, there would be deterioration in our patient’s metabolic status, in the absence of oestrogen treatment.
The phenotype of the CYP19A1 knockout (ArKO) mouse, developed from a disruption to exon 9, provides further evidence for an association in humans between aromatase, and hence oestrogen, deficiency, and hyperandrogenism, and visceral obesity and metabolic alterations [41, 42]. With increasing age, both male and female ArKO mice, had progressive accumulation of more intra-abdominal adipose tissue than the wild-type [43]. This was accompanied by a reduction in lean body mass in the ArKO mice such that overall weight was unchanged [43]. Increased adiposity was associated with reduced spontaneous physical activity, but not with hyperphagia or reduced resting energy expenditure [43]. Administration of oestrogen resulted in a reduction in adipose depot size to one comparable with that of wild-type, directly implicating oestrogen deficiency in the pathogenesis of visceral obesity [43]. Elevated cholesterol, insulin levels, glucose intolerance and hepatic lipid accumulation have also been noted in the ArKO mice [43, 44]. Oestrogen treatment improved glucose tolerance and reversed hepatic steatosis in male ArKO mice [44, 45]. Thus, the majority of the available clinical and animal data overwhelmingly infer that oestrogen deficiency whether due to aromatase deficiency, the menopause or target tissue resistance, is associated with a phenotype of metabolic syndrome.

7) In the discussion, please take into consideration what your results mean in terms of natural history of the disease and the disease duration needed to develop bone and metabolic alterations.

We have mentioned that both bone and metabolic alterations develop after long-term oestrogen deficiency (Page 8, Line 219-220; Page 10, Line 273-5).

Minor comments

1) Introduction (page 4; lines 92-93): when speaking about the twenty-four cases please refer also to the recent extensive review in men (Rochira & Carani, Nature Reviews Endocrinology, 5:559–568, 2009) and use the term ‘about 24 cases’ since recently other few cases have been described in endocrinological congresses (ENDO 2012, ENDO 2013, ECE 2013). The cases are increasing and I think that the number you cite under esteem the real number of cases.

We have rephrased this as “approximately twenty-four cases” (Page 4, Line 91).

2) Case Presentation (page 6, lines 166-171). A table will be of help for showing all the results of biochemical examinations.

We thank the reviewer for this suggestion. We have presented the results of biochemical investigations in Table 1.

3) Had the patient bone pain? Other skeletal deformities (e.g. scoliosis, necrosis of the femoral head, genu valgum)?

The patient did not have bone pain or any clinically apparent skeletal deformities.

Reviewer 3: Arundhati Sharma
Reviewer's report:
In the paper entitled “Streak ovaries in association with Aromatase deficiency due to a novel CYP19A1 mutation”. The authors have reported a novel duplication in CYP21 gene in a patient with aromatase deficiency. They have also tried to show the effect of this duplication on the alpha helix of the protein and how it would affect the binding.

The following points may be addressed by the authors ……..

Major revision.
The paper needs to be revised and presented in a concise manner. Since the authors have examined the patient at the age of 32 years and did not have a chance to review any of her previous records or slides, a short past clinical history may be presented without actually quoting any records.

The authors may emphasize on the clinical examination and hormonal investigations undertaken by them. The presence of streak gonads should not be over stressed as this might be misleading, especially for future research studies because the initial diagnosis of the patient was incorrect; diagnosis of Turner syndrome with 46, XX/45, X and Y chromosome mosaicism with an unusually tall stature is doubtful- that too at an age of 25 years. The streak gonads observed on USG might support the diagnosis of Turner syndrome but it may be little premature to conclude without any further evidence that aromatase deficiency led to streak gonads in this patient. The title may be modified….. for instance to “association of a novel CYP19A1 duplication mutation with Aromatase deficiency”

We thank the reviewer for these comments. We have reduced the detail presented in the past clinical history, retaining only what we believe are the most salient and relatively indisputable aspects. We have shifted the emphasis to the clinical examination and hormonal investigations that we have undertaken, as requested. Whilst this reviewer has requested that we present the paper in a more concise manner, other reviewers have asked for more detail in several areas. We have tried to be as concise as possible, whilst also addressing the issues, additional points and discussion that have been requested by other reviewers.

We have revised the title of the manuscript as requested. The manuscript is now entitled: “A case of Aromatase deficiency due to a novel CYP19A1 mutation”

We have placed lesser emphasis on the streak ovaries as requested. We would like to clarify that these were noted not on ultrasonography, but at laparoscopy. There is no single ovarian phenotype in patients with aromatase deficiency – indeed the reported morphology has varied from normal to multicystic. We surmise from the ovarian histology report from our patient, that she did not have obviously enlarged, multicystic ovaries as described in some cases. Following treatment with oestrogen, regression of ovarian cysts has been documented in
several case reports and it may be that the appearance of the ovaries reported in our patient was a consequence of oestrogen treatment. We have elaborated on this in the manuscript (Page 11, Lines 298-313 and Page 12, Lines 314-25). We reproduce our discussion below for your reference:

Unexpectedly, our patient was reported to have had streak ovaries excised. We caution that the histological slides were unavailable for us to verify the pathology report especially since streak ovaries have not been previously described in association with aromatase deficiency. Polycystic ovaries have been described, consistent with the increased androgenisation in tissues [18-22]. Regression of ovarian cysts with oestrogen treatment has been described in several cases [19, 20, 22, 49]. Other case reports however have described normal ovarian morphology, even in the absence of prior oestrogen treatment [10, 11]. Thus, the case reports to date suggest that there is no consistent ovarian phenotype in aromatase deficiency and that the phenotype may be modulated by treatment. Hence, in our patient, the ovarian histology may be a result of oestrogen treatment.

Alternatively, extrapolating from observations in the ArKO mouse model, we speculate that the streak ovaries may be an inherent manifestation of CYP19A1 deficiency [41, 42]. Follicular development in ArKO is abnormal in an age dependent manner, with an early block in follicular development at the antral stage with absent corpora lutea, then haemorrhagic cysts and later, absent secondary and antral follicles and atresia of primary follicles with increased collagen deposition [41, 42]. Hence, whilst not previously reported, it is possible that the longer life span of the human may permit more complete follicular atresia and collagen deposition mimicking the classical streak ovaries seen in Turner’s syndrome. Streak ovaries may not have been previously described in human CYP19A1 deficiency because direct visualization of the ovaries with laparoscopy would be infrequently performed and some of the cases reported may have been too young to develop this manifestation. In addition, some of the cases have had CYP19A1 mutations that allowed some residual aromatase function and may have a less severe ovarian atretic phenotype.

Another possibility is that our case may harbour another cause of the streak ovaries such as XO mosaicism localised to the ovaries, although the normal XX chromosomal composition in both lymphocytes and in fibroblasts makes this less likely. Streak ovaries are not clearly associated with any other disorder but Turner’s syndrome. In view of the small numbers of CYP19A1 cases reported this manifestation should be noted particularly since this finding may be relevant to ovarian developmental biology.

The authors may look up the exact way of reporting a mutation. The transcript reference etc. need not be mentioned in the results. This can be provided in supplementary material.

We have amended the reporting of the mutation, consistent with the recommendations of the Human Genome Variation Society and as discussed by
den Dunnen and Antonarakis in their paper “Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion” in Human Mutation 2000; 15:7-12; which is still the paper referenced by the Human Genome Variation Society as at September 13, 2013. We have reported the mutation as p.Ala306_Ser314dup; and have provided full details in the supplementary material as suggested by the reviewer.

1) Abstract-

Background: Line 1: the line may be changed to about 24 case reports.
This sentence has been amended as requested (Page 2, Line 38).

Line 4: this sentence may be changed to “We report a case of
.............diagnosed at the age of ....years (age may be mentioned) due to a
novel duplication..............gene.
This sentence has been amended as requested (Page 2, Line 42).

Case presentation: The gene symbol of aromatase gene, MIM Number may be
mentioned. Exon nomenclature may be in ...Arabic ...numerals. Mention first the
exon and then the duplicated region.
This sentence has been amended as requested (Page 2, Lines 50-51).

Conclusion: May be revised to make more precise. Sentences like “few cases of
aromatase ......” and “this may reflect few adult case” are repetitive and may be
omitted.
This has been revised as requested (see Conclusion, Page 2).

2) Main Manuscript-

Background: Paragraph 2 line 1: MIM nomenclature of CYP21 may be provided;
coding exons should be numbered in Arabic numerals.
MIM nomenclature has been provided as requested; coding exons have been
numbered in Arabic numerals (Page 3, Line 83; Page 3, Line 84; respectively).

Line 10: Up until recently .....exons 3rd-6th, this sentence is not clear.
This sentence has been revised to clarify that up until recently all mutations
reported were in coding exons. Whilst most mutations have been in exons 9 and
10, mutations in exons 3-6 have also been reported. Furthermore a promoter
variant has been reported recently which has been deemed to have functional
significance. We have elaborated on this in the text (Page 5, Lines 124-135), and
the amendment is shown below for your reference.

Up until recently, all reported mutations have been in coding exons [9]. Whilst
most mutations are in exons 9 and 10, encoding the substrate (androgen)
binding site and haem-binding domain, respectively, mutations have also been
reported in exons 3 to 6 [9]. The mutations result in amino acid substitutions,
premature stop codons or altered exon-intron splice sites [9]. Recently, a patient
with aromatase deficiency was found to be heterozygous for a novel
non-synonymous mutation (p.Asn411Ser) in exon 9 [10]. In vitro studies confirmed this was a loss-of-function mutation; when co-expressed with the wildtype protein, the enzyme activity of the mutant protein was approximately 65% of wildtype, compatible with a recessive disease. Thus this mutation alone was considered insufficient to explain the observed phenotype. Sequencing of the placental promoter I.1 revealed a heterozygous, paternally inherited C>T variant -41 base pairs upstream of exon 1, a previously observed polymorphism (rs6493497) [10, 16]. In vitro studies revealed a 50% reduction in transactivation ability of the placental promoter harbouring the mutation, compared with wildtype [10].

Line 13: ‘Recently a patient with…………in exon 10’. The single base pair change at position -41 may be specified. Is this a SNP? Has it been reported? Its role in reduction of aromatase levels may be mentioned.

We have reported the single base pair change. This is a reported SNP (we have provided the rsID in the paper). We have described its role in reduction of aromatase levels (Page 5, Lines 128-135). The amendment is shown below for your reference.

Recently, a patient with aromatase deficiency was found to be heterozygous for a novel non-synonymous mutation (p.Asn411Ser) in exon 9 [10]. In vitro studies confirmed this was a loss-of-function mutation; when co-expressed with the wildtype protein, the enzyme activity of the mutant protein was approximately 65% of wildtype, compatible with a recessive disease. Thus this mutation alone was considered insufficient to explain the observed phenotype. Sequencing of the placental promoter I.1 revealed a heterozygous, paternally inherited C>T variant -41 base pairs upstream of exon 1, a previously observed polymorphism (rs6493497) [10, 16]. In vitro studies revealed a 50% reduction in transactivation ability of the placental promoter harbouring the mutation, compared with wildtype [10].

Line 16: uniformity may be maintained in the style of writing mutations throughout the paper- either a three letter code like Asn 411Ser or N 411S may be adopted.

We have amended the paper to maintain uniformity of writing mutations.

Paragraph 3, lines 30-33. ‘Many of the phenotypic……development does not add any new information and may be omitted.

We have removed this statement as requested.

Case presentation- paragraph2, line 3…..’She disguised ….loose garments’ this sentence may be omitted.

We have removed this statement as requested.

Paragraph 3, line 1…age 25 years to be added.
Reference to her age has been added as requested (Page 6, Line 155).

Line 6-12….the past reports may be just reported in a short concise way without
“quoting”.
We have amended this statement (Page 6, Lines 154-163).

Paragraph 7, line 47-49: ‘Most individual ……….reported’. This sequence does not seem relevant here.

We have moved this statement to paragraph 5 of the “Background” section of the paper where we discuss the previous case reports of Aromatase deficiency and mutations (Page 5, Lines 124-35). We feel this sequence is more appropriately placed in the paper now.

Were parents of the patient tested for the mutation? That would provide relevant information on the carrier status of the parents.

The parents of the patient were not tested for the mutation. They live in rural India and were not available for testing. We have made reference to this (Page 7, Lines 192-193).

Paragraph 9, line 68 (reference may be given here)

We have added references to the studies of the Aromatase knockout mouse performed by Britt et al., references 41 and 42.

Conclusion: May restrict to reporting just the novel duplication mutation. The associated clinical parameters observed by the authors are reported in other cases.

We have amended the conclusion as follows (Page 12, Lines 328-34):

We report a case of the very rare condition, aromatase deficiency, due to a novel mutation and corresponding enzyme region distinct from previous reports. Our case is unique because it is the first reported case of aromatase deficiency in a female not treated until adulthood, and it depicts, for the first time, the natural history of aromatase deficiency in females. This comprises a phenotype of osteopaenia, tall stature and metabolic syndrome, as occurs in aromatase deficient males in whom, similarly to our patient, diagnosis and treatment are delayed. Streak ovaries may represent an extension of the clinical spectrum of this disorder and appears to correlate with findings in the aromatase knockout mouse model.

The Authors may reconsider inclusion of Figure 1
We have reconsidered inclusion of Figure 1. At this stage, we are keen to retain it in the manuscript.