Original Investigation

**Assessment of the ovarian reserve in patients with Beta-thalassemia major; A prospective longitudinal study**

Özcan et al. Ovarian reserve in patients with Beta-thalassemia major

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**Abstract**

**Objective:** Repeated blood transfusions in women with Beta-thalassemia major (BTM) may lead to iron overload and increase oxidative stress and consequently resulting in ovarian damage. We aimed to evaluate the alterations in the ovarian reserve of transfusion-dependent BTM patients over a time period of one year and compared the anti-mullerian hormone (AMH) levels between women with BTM and healthy population.

**Material and Methods:** This longitudinal prospective study was conducted in 41 women with transfusion-dependent BTM at the tertiary hospital. The hospital database between 1996 to 2021 was screened for women diagnosed with BTM. Anti-mullerian hormone levels were assessed at baseline and one year later.

**Results:** Twenty-five (60.9%) of the patients had amenorrhea while 16 (39.1%) were observed to have normal cycles. The mean AMH values of all women were 2.7±1.8 ng/mL at the initial measurement which was significantly lower compared to age related AMH (mean 4.0±0.4 ng/mL) nomogram of healthy population (P= 0.001). The baseline AMH values of the patients with amenorrhea were found to be lower than the patients with normal menstrual cycles (2.1±1.8 vs 3.6±1.5 ng/mL, P= 0.009). After a one-year follow-up, there was a trend towards a decrease in AMH levels of the patients with normal menstrual cycles.

**Conclusion:** Serum AMH values is decreased in patients with transfusion-dependent BTM. It would be beneficial to inform all patients about possible effects of repeated blood transfusions on fertility.

**Keywords:** Ovarian reserve, beta thalassemia, anti-mullerian hormone
Introduction
Beta-thalassemia is the most common major monogenic hemoglobin disorder worldwide (1). Deficiency of β-globin chain synthesis is a condition that causes decreased production of red blood cells and hemoglobin resulting in severe anemia (1,2). The disease that was previously life-threatening is now treated with blood transfusion therapy and iron chelation therapy providing a decrease in morbidity and mortality. However, repeated blood transfusions may lead to an increase in reactive oxygen species over years due to excessive production of free radicals and pro-oxidant/antioxidant imbalance with iron overload in those patients (3,4). The accumulation of hemosiderosis in the hypothalamus and pituitary gland as a result of repeated blood transfusions may cause hypogonadotropic hypogonadism in most women (2,5). It is the most common endocrinopathy in thalassemia patients (40-91%) leading to sexual dysfunction and subfertility (6-8). Hypothalamo-pituitary damage is usually irreversible (2) and hypogonadotropic hypogonadism appears to be common despite advances in chelation regimens (6,9).
Anti-mullerian hormone (AMH), secreted by granulosa cells of preantral and antral follicles, is a member of the transforming growth factor-β family (5,10) and its levels do not change throughout the menstrual period. It is the most reliable biochemical marker showing ovarian reserve. AMH levels are widely used in the studies to show the extent of ovarian damage after the use of gonadotoxic agents in women, particularly in women who received chemotherapy after cancer diagnosis.
It is still unclear whether ovarian reserve is intact in patients with Beta-thalassemia major (BTM) despite chronic blood transfusion and hypogonadotropic hypogonadism secondary to hemosiderosis. In this prospectively designed study, we first aimed to evaluate the alterations in the ovarian reserve of BTM patients over a time period of one year and compared the AMH levels between women with BTM and healthy population. Secondly, we further divided patients with BTM into two groups as amenorrheic and those with normal cycle, and aimed to compare the ovarian reserve between these groups.

Materials and methods

Patient population
This longitudinal prospective study was conducted in 41 women with transfusion-dependent BTM at the tertiary hospital. The hospital database between 1996 to 2021 was screened for women diagnosed with BTM. In those women, the transfusion regimens allowed hemoglobin levels to be maintained between 9.5 and 14.0 g/dL. Women were transfused at 14-28 day intervals. Chelation was initiated after two years of transfusion or when the serum ferritin level was consistently higher than 1000 mg/L. The efforts were made to reduce ferritin levels below 1000 mg/L. All patients received chelation therapy. Deferasirox (maximum 40 mg/kg/d) and deferiprone (40 mg/kg/d) were administered orally alone or in combination. Deferoxamine was administered at a dose of 25-40 mg/kg/5–7 d in combination with
deferasirox or deferiprone. After verbal and written information about the study, all eligible
and voluntary participants gave informed consent. Exclusion criteria were the presence of any
comorbid systemic condition, prior chemotherapy, hormonal therapy or immunotherapy and
previous ovarian surgery. This study was approved by our local ethics committee (Dated
24.02.2021 and IRB: 0044) and conducted according to the principles of the Declaration of
Helsinki.

Serum iron and ferritin levels of the patients were tested. Anti-mullerian hormone levels were
measured at admission for the study after ethical approval and at the follow-up one year later.
The volume of the ovaries and the number of antral follicles were evaluated using ultrasound
by the same gynecologist. Antral follicle count measurements were performed using high-
resolution transvaginal ultrasonography during the early follicular phase. The results of
follicle stimulating hormone (FSH), luteinizing hormone (LH), and estradiol hormone used to
assess the pituitary and gonad functions of the patients were noted. Hypogonadotrophic
hypogonadism (HH) was defined as FSH and LH levels <2 iu/l with accompanying E2 levels
<20 pg/ml (11). The history of menstrual regularity was obtained by self-report. The blood
to sample taken for pre-transfusion biochemistry examinations of all cases were collected from
the biochemistry laboratory after the examination, and two blood serum samples collected
from female patients at the baseline and 12 months were stored at -80°C until evaluation.

*Amh measurement*

Serum AMH levels were analyzed using the Elecsys AMH Plus test with AMH assay on the
Cobas-E electrochemiluminescence immunoassay platform (Roche Diagnostics GmbH,
Mannheim, Germany). Serum AMH values were reported as ng/mL and the assay range was
0.01–23 ng/mL. The intra and inter-assay variation coefficients were <8% and <12%,
respectively.

**Outcome measures**

Our primary outcome was to assess AMH levels after a one-year follow-up and to compare
AMH levels of patients with BTM to healthy population. Our secondary aim was to compare
the characteristics and AMH values of the patients when women with BTM were divided
according to their menstrual status. For the main purpose of the study, patients were followed
for one year. Although the pregnancy outcomes were not the main purpose of our study, the
information of the patients who continued to be followed up and who became pregnant over
the years or who received assisted reproductive therapy were also included in the study.

**Statistical analysis**

Mean and standard deviation values of measurable variables were calculated. The data
distribution normality between dependent sample groups was evaluated using Smirnov-
Kolmogorov analysis. Of the dependent variables, the Paired-t test was applied to normally
distributed data while the Wilcoxon test was used for non-normally distributed data.
Categorical variables were compared using the χ² test. The one-sample t-test was used to
determine whether AMH levels of women with BTM differs from the AMH levels of healthy
Turkish population. The correlations between AMH levels and ferritin values were calculated
using Pearson’s correlation analysis. All statistical analyses were performed with SPSS
(Statistical Package for the Social Sciences) 18.0 Microsoft for Windows program. A p value
of <0.05 was accepted statistically significant.

**Results**

Forty-one patients with BTM were included in the study. The mean age of all women were
23.0±5.7 years. Twenty-five (60.9%) of the patients had amenorrhea while 16 (39.1%) were
observed to have normal cycles. The mean age (24.4±5.9 years vs 20.8±4.9 years; p= 0.061 in
the amenorrheic and normal cycle groups, respectively) and body mass index (21.8±1.8 vs
21.9±1.9 kg/m²; p= 0.781 in the amenorrheic and normal cycle groups, respectively) were
similar between two groups. The clinical characteristics of all patients included in the study were given in Table 1.

The mean AMH values of all women were 2.7±1.8 ng/mL at the initial measurement which was significantly lower as compared to age related AMH (4.0±0.4 ng/mL) nomogram of healthy population published elsewhere (p= 0.001) (12). The mean AMH levels of all women were evaluated after one year to find out whether there was a significant decline in AMH levels in a short time period. The mean AMH levels were found to be lower compared to those measured at baseline however the result was not significant (2.7±1.8 ng/mL for mean initial AMH, 2.5±1.8 ng/mL for mean control AMH; p= 0.207).

We further evaluated the ovarian reserve of women with BTM according to their menstrual status. The mean number of antral follicles in the amenorrhea group was 8.6 ± 5.7 while that was 12.0±2.3 in patients with normal cycles (p= 0.048). Mean ovarian volume was calculated as 5.5±4.4 cm³ and 10.9±5.7 cm³ in patients with amenorrhea and normal cycle, respectively (P= 0.003). Initial AMH values of patients with amenorrhea were found to be lower than those in patients with normal menstrual cycles (2.1±1.8 vs 3.6±1.5 ng/mL, respectively; p= 0.009).

FSH and LH values of amenorrheic patients were found to be lower than normal cycle patients (Table 2). After a one-year follow-up, there was a trend towards a decrease in AMH levels in normal cyle group and statistically significance remained between patients with amenorrhea and normal cycle (2.1±1.8 vs 3.3±1.5, respectively; p= 0.031). The comparison between clinical features of women with amenorrhea and normal cycle regarding was given in Table 2.

In the correlation analysis, a significantly inverse correlation was found between AMH levels and ferritin levels both at baseline (r= -0.486, p= 0.004, Figure 1A) and one year later (r= -0.488, p= 0.003, Figure 1B). Ferritin levels were higher in amenorrheic patients than the patients with normal menstrual cycle (p=0.041).

Although pregnancy outcome of women with BTM was not one of the main outcome measures of this study; 22 of the 41 female patients were married. Twenty patients were married with healthy partners whereas others (n=2) were married with partners with BTM. Out of 22 women, 10 women were amenorrheic whereas 12 had normal menstrual cycle. Six of the 12 patients with normal menstrual cycle (50%) gave birth to 7 healthy babies without receiving any treatment. Out of 12 women with amenorrhea, 4 women underwent in vitro fertilization (IVF) procedure. Only one of them achieved pregnancy and had a live birth while IVF treatment failed in 3 women. Six women did not have a desire for pregnancy and had not started any infertility treatments yet.

Discussion
In this prospectively designed study, we investigated the effects of repeated blood transfusions on AMH levels at baseline and after a one-year follow-up in women with BTM and found that women with BTM had lower AMH levels as compared to healthy population, and there was a trend towards a decrease in AMH levels after a one-year follow-up. The presence of amenorrhea seems to be mainly related with hypothalamic dysfunction, rather than ovarian reserve. However, we demonstrated that longer duration of blood transfusions may lead to hypogonadotrophic hypogonadism resulting in lower AMH levels and decreased ovarian volume compared to women with regular cycles. To our knowledge, this is the first prospective longitudinal study demonstrating the alteration in ovarian reserve of women with BTM after a one-year follow-up.

Hypogonadotropic hypogonadism (70-80%) and amenorrhea due to hemosiderosis of the hypothalamus and pituitary gland are common in BTM patients (2,11,13), however, the effect of iron load on the ovaries is unclear (14-16). There are only limited data available in the literature researching ovarian reserve in patients with BTM (2,5,9,10), In a study conducted with 17 BTM patients (9 amenorrheic, 8 with normal menstrual cycles), the mean age of the
patients was found to be 33.8±5.6 years (2). AMH values of BTM patients were found to be significantly lower than the control group and those of the amenorrheic group were found to be lower than those of patients with normal menstrual cycles. In our cohort, the frequency of amenorrhea is 60.9% in BTM patients. In another study, 29 BTM patients with a mean age of 21.4±5.8 years and 29 control patients with a mean age of 21.5±6.2 years were evaluated in terms of ovarian reserve. Although AMH values were found to be lower in the BTM group compared to the control group, the result was not significant (5). Singer et al. evaluated 26 patients with BTM and compared AMH levels between women with amenorrheic (29±5 years) and normal menstrual cycles (27±8) and they concluded that there was no significant difference between two groups in terms of AMH values and antral follicle numbers (9). All the studies above mentioned were retrospective and study populations were small (2,5,9).

Uysal et al. compared AMH values between BTM patients (n=43) and healthy controls (n=44) in a prospectively designed case-control study (10). The mean age of patients with BTM was 23.4±5.1 years. The median AMH values (1.7 vs 3.5 ng/ml; P= 0.002, respectively) and median antral follicle numbers (3 vs 11; p< 0.001, respectively) were found to be lower in the BTM group compared to the control group. When subgroup analysis was performed among BTM patients, it was found that the AMH and antral follicle count values of amenorrheic patients were lower than those of patients with normal menstrual cycles. Since women with normal menstrual cycles were younger than the amenorrheic group and they had a shorter duration of transfusion therapy, it is reasonable that they had better ovarian reserves (10). Similar to the study by Uysal et al, the amenorrheic group had lower AMH levels, antral follicle numbers and mean ovarian volumes in our study. Given that there are studies reporting that AMH is not influenced by hypothalamic amenorrhea (17,18), significantly decreased AMH levels in women with amenorrhea may be due to the direct gonadotoxic effects of iron overload.

In a study that examined the patients with BTM who received chelation therapy for 5 years, high serum ferritin level, poor compliance with chelation therapy and early-onset transfusion therapy were found to be the main risk factors associated with endocrine complications. The same study also reported that serum ferritin levels of 2000 ng/mL were related with hypogonadism (8). In another study by Chang et al., ferritin levels were found to be significantly higher in patients with hypogonadotropic hypogonadism than those without hypogonadotropic hypogonadism (5). In addition, a negative correlation was found between ferritin and AMH levels (5,10). It is concluded that high ferritin levels may lead to ovarian damage as well as the dysfunction in hypothalamo-pituitary axis. Although women with HH may have lower AMH levels due to long-term ovarian suppression (11,17,18), AMH level is still one of the best predictors of controlled ovarian stimulation in those women reported by many studies (11,18). In our study, a significantly inverse correlation was found between the AMH and the ferritin levels tested both at baseline and one-year follow-up. Consistently with other studies (10), ferritin levels were demonstrated to be higher in amenorrhea patients than patients with normal menstrual cycles.

It can be questioned whether pregnancy and fetal outcomes are comparable between the healthy population and patients with BTM. Cesarean section is frequently preferred in women with beta-thalassemia, and infant weight at birth is found to be lower than the general population despite term delivery (19). Spontaneous abortion, fetal loss, preterm delivery, fetal growth restriction and low birth weight are more common in pregnant women with BTM compared to healthy women (20). In the present study, we reported a few number of pregnancies and all terminated with term birth without any fetal and obstetric complications. One of the limitations of this study was the lack of control group however the presence of age related AMH nomogram of Turkish population in the literature was useful to determine the difference between AMH levels. Furthermore, when we limited the study to the patients with
regular menstruation, there were no difference in AMH levels as compared to healthy population that may be due to the inclusion of relatively younger population and shorter follow-up period. Therefore, further research with longer follow-up period should be warranted to draw more definite conclusions regarding the possible time period in which the iron accumulation initiates ovarian damage and to distinguish hypogonadotrophic hypogonadism-related low AMH levels from the direct ovarian damage-related low AMH levels.

Conclusion
The AMH values of transfusion-dependent BTM patients decreased significantly over time and the frequency of amenorrhea increased with advanced age. It would be beneficial to inform all patients regarding possible effects of repeated blood transfusions on fertility. Further research is required to understand the extent of ovarian damage in advanced aged BTM women with hypogonadotropic hypogonadism.

References

Table 1. The clinical characteristics of all patients with Beta-thalassemia major

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients (n=41)</th>
</tr>
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<tbody>
<tr>
<td>Age, years</td>
<td>23.0±5.7</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>21.8±1.8</td>
</tr>
<tr>
<td>FSH, mIU/mL</td>
<td>3.1±2.4</td>
</tr>
<tr>
<td>LH, mIU/mL</td>
<td>2.9±2.9</td>
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<tr>
<td>E2, pg/mL</td>
<td>58.9±129.4</td>
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<tr>
<td>Initial AMH levels, ng/ml</td>
<td>2.7±1.8</td>
</tr>
<tr>
<td>AMH levels at 12 month, ng/ml</td>
<td>2.5±1.8</td>
</tr>
<tr>
<td>AFC</td>
<td>9.9±4.9</td>
</tr>
<tr>
<td>Mean ovarian volume, mm³</td>
<td>7.6±5.5</td>
</tr>
<tr>
<td>Mean uterine volume, cm³</td>
<td>29.2±23.5</td>
</tr>
<tr>
<td>Initial ferritin levels, ml/ng</td>
<td>2208±1811</td>
</tr>
<tr>
<td>Ferritin levels after one year, ml/ng</td>
<td>2280±2040</td>
</tr>
</tbody>
</table>

Table 2. Comparison between the clinical features of patients with amenorrhea and normal cycle

<table>
<thead>
<tr>
<th></th>
<th>Amenorrhea (n=25)</th>
<th>Normal cycle (n=16)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>24.4±5.9</td>
<td>20.8±4.9</td>
<td>0.061</td>
</tr>
<tr>
<td>Years receiving transfusions</td>
<td>20±5</td>
<td>18±5</td>
<td>0.095</td>
</tr>
<tr>
<td>BMI, kg/m2</td>
<td>21.8±1.8</td>
<td>21.9±1.9</td>
<td>0.781</td>
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<tr>
<td>FSH, mIU/mL</td>
<td>2.5±2.3</td>
<td>4.2±2.1</td>
<td>0.040</td>
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<tr>
<td>LH, mIU/mL</td>
<td>1.7±1.5</td>
<td>4.7±3.6</td>
<td>0.001</td>
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<tr>
<td>E2, pg/mL</td>
<td>16.4±14.2</td>
<td>125.7±121.2</td>
<td>0.011</td>
</tr>
<tr>
<td>Initial AMH levels, ng/ml</td>
<td>2.1±1.8</td>
<td>3.6±1.5</td>
<td>0.009</td>
</tr>
<tr>
<td>AMH levels at 12 month, ng/ml</td>
<td>2.1±1.8</td>
<td>3.5±1.5</td>
<td>0.031</td>
</tr>
<tr>
<td>AFC</td>
<td>8.6±5.7</td>
<td>12.0±7.1</td>
<td>0.048</td>
</tr>
<tr>
<td>Mean ovarian volume, mm³</td>
<td>5.5±4.4</td>
<td>10.9±5.7</td>
<td>0.003</td>
</tr>
<tr>
<td>Mean uterine volume, cm³</td>
<td>19.1±19.0</td>
<td>45.0±21.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Initial ferritin levels, ml/ng</td>
<td>2742±2019</td>
<td>1745±1155</td>
<td>0.041</td>
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<tr>
<td>Ferritin levels after one year, ml/ng</td>
<td>2962±3785</td>
<td>1400±1066</td>
<td>0.032</td>
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Figure 1. A) Correlation curve of initial AMH and ferritin values at baseline B) Correlation curve of AMH and ferritin values one year later