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Original article

Retrospective study on long-term effects of hormone replacement therapy (HRT) and iron chelation therapy on glucose homeostasis and insulin secretion in female ß- thalassemia major (β-TM) patients with acquired hypogonadotropic- hypogonadism (AHH)

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Abstract. *Background and aim:* Hypogonadism and abnormalities of glucose homeostasis, resulting from iron-induced pituitary and pancreatic β-cell dysfunction respectively, are the most frequently reported endocrine abnormalities in patients with ß-thalassemia major (β-TM), also identified as transfusion-dependent thalassemia (TDT). *Study design and Patients:* The aim of the present retrospective study was to evaluate the long-term effects of hormone replacement therapy (HRT) on glucose metabolism and insulin secretion/sensitivity during 3-h oral glucose tolerance test (OGTT) in adolescent and young β-TM women with acquired hypogonadototropic -hypogonadism (AHH). Twelve hypogonadal β-TM females with AHH on HRT were followed for 8.26 ± 1.49 years. *Results:* At baseline, 10 patients (83.3%) had normal OGTT, 1 patient presented with impaired glucose tolerance (IGT) and 1 patient had an isolated PG level of 165 mg/dL at 1-h during OGTT (H-NGT). At last evaluation, 7 patients (58.4%) had normal OGTT, while 5 patients (41.6%) had abnormal OGTT. Reduced insulin sensitivity and impaired first-phase insulin secretion were also documented. Three of 4 β-TM patients on treatment with estradiol hemihydrate MX 50 patches plus oral medroxyprogesterone acetate (MPA), associated with a very effective iron chelation therapy, maintained normal glucose tolerance from baseline to last evaluation. Significant adverse events due to HRT or additional endocrine complications were not documented in any cases during the follow-up. *Conclusion:* Deterioration of glycemia (dysglycemia) occurred in 45.4% (5/11) of thalassemic females on long-term HRT. Additional studies are needed to elucidate the validity of our preliminary observations. (www.actabiomedica.it)

Key words: ß- thalassemia major, acquired hypogonadotropic- hypogonadism in females, hormone replacement therapy, oral glucose tolerance test, glucose homeostasis, iron overload

Introduction

Hypogonadism and abnormalities of glucose homeostasis, resulting from iron-induced pituitary and pancreatic β-cell dysfunction respectively, are the most frequently reported endocrine abnormalities in patients with ß-thalassemia major (β-TM), also identified as transfusion-dependent thalassemia (TDT) (1).

In females with β-TM, acquired central hypogonadotropic hypogonadism (AHH) is characterized by delayed puberty or pubertal arrest, absent sexual development (primary amenorrhea) or secondary amenorrhea associated with inappropriately low gonadotropins (LH and FSH) and sex steroids (estradiol) levels, in the absence of anatomical abnormalities of the hypothalamic-pituitary-gonadal (HPG) axis (2).

The aims of therapy in hypogonadal adolescents or young adults are to induce normal pubertal development, to avoid the long-term health consequences on bone health, to alleviate psychological morbidity (excessive shyness, social anxiety, delayed sexual debut, and decreased marriage rate), and to induce fertility on patient request (2-4). Although the therapeutic objectives of hypogonadotropic hypogonadism are well defined, the relevant literature focusing on AHH in adolescents and young adults with β-TM and AHH is limited. Moreover, most therapeutic regimens are not evidence-based and are transposed from guidelines prepared for patients with hypergonadotropic hypogonadism, such as Turner syndrome (TS) and premature ovarian insufficiency (POI) (2).

Hormone replacement therapy (HRT) in β-TM patients is extremely complex because of associated co-morbidities, such as iron overload, thrombophilia, chronic liver disease, gallbladder disease, impaired glucose tolerance or diabetes, and cardiomyopathy (5-9).

In a long-term retrospective study over 40 years in a cohort of 42 patients, 6/42 (14.2%) splenectomized β-TM female patients on HRT had adverse reactions (ADR): 2/42 patients (4.7%) had an episode of transient monocular visual loss and a stroke with right hemiparesis; 4/42 (9.5%) manifested a deterioration of glucose homeostasis [3 patients (7.1%) from normal oral glucose tolerance test (NGT) to impaired glucose tolerance (IGT) and 1 (2.3%) from IGT to diabetes]. Mild elevation of liver enzymes and bilirubin occurred in 3/42 (7.1%) female patients (6).

The aim of the present retrospective study was to evaluate, in adolescents and young β-TM women with AHH, the long-term effects (mean duration follow-up: 8 years, range: 6.3-10.4 years) of HRT on glucose metabolism and insulin secretion/sensitivity, during a 3-h oral glucose tolerance test (OGTT).

Subjects and methods

A. Study population and design

From October 2010 to January 2023, twelve adolescent and young, adult female β-TM patients with different clinical manifestations of AHH (arrested puberty, primary or secondary amenorrhea) were referred for consultation and follow-up to an Italian centre, the Pediatric and Adolescent Outpatient Clinic, Private Accredited Quisisana Hospital, Ferrara, Italy.

The diagnosis of AHH was based on the following criteria: low basal plasma estrogen level in association with low luteinizing hormone (LH) and follicle-stimulating hormone (FSH), at baseline and after stimulation with gonadotropin-releasing hormone (Gn-RH stimulation test), associated with a negative medroxyprogesterone acetate test (MAP test: 10 mg given orally for 7 days) in the presence of a normal serum prolactin (PRL) and thyroid stimulating hormone (TSH) level.

Arrested puberty (AP) was defined as the lack of pubertal progression for more than 3 years after spontaneous breast bud onset. Primary amenorrhoea (PA) was defined as the absence of menarche at the age of 16 years. Secondary amenorrhea (SA) was defined as the absence of menstruation for 6 months and over at any time after menarche in β-TM patients in whom systemic or functional causes had been ruled out (11).

Exclusion criteria were: a) non-transfusion-dependent thalassemia (NTDT); b) β-TM patients with poor or inconsistent compliance to HRT; c) bone marrow transplanted patients; d) patients with HIV positivity; e) patients with depression, anxiety disorders or eating disorders; f) patients with chronic

kidney diseases, history of severe head injury, autoimmune diseases or history of chemo-or radiotherapy.

HRT was prescribed either orally or transdermally (TD). Before treatment, a careful history of past and present medical conditions (positive personal history of a thromboembolic event, chronic liver disease and gallbladder disease, nicotine abuse, drug abuse), and family history of thromboembolism in a first-degree relative. Physical examination (presence of obesity, or hypertension), and laboratory assessments (complete blood count, coagulation and thrombotic assays) were done to exclude conditions or risk factors that could represent a contraindication to treatment.

B. Anthropometry and assessment of associated endocrine complications

Height and weight were assessed and evaluated according to Italian growth charts for height, weight and Body Mass Index (BMI) (12,13). Short stature was defined as height 2 SD below the mean height for age and sex. BMI was evaluated based on the World Health Organization (WHO) recommendations: underweight (<18.5 kg/m^2); normal range (18.5–24.9 kg/m^2); overweight (25.0–29.9 kg/m^2); obese (≥ 30 kg/m^2) (14). Associated endocrine complications were assessed and defined according to the I-CET position statement published in 2013 (13). The circulating concentrations of LH, FSH, prolactin and estradiol (E2), free thyroxine (FT4), thyrotropin (TSH), and cortisol were measured by commercial kits using automated chemiluminescence immunoassays.

Procedures

A. Oral glucose tolerance test (OGTT)

A standard OGTT (max 75 g of glucose) was performed in the morning, after an overnight fast, in subjects who were clinically stable and without a history of acute infection in the previous 3 weeks. Venous blood samples were collected at 0, 30, 60, 90, 120 and 180 min after glucose load for plasma glucose (PG) and insulin assay. Glucose tolerance was classified in accordance with the American Diabetes Association criteria (15). An isolated 1-hour post-load PG value ≥155 mg/dl (H-NGT) was considered an additional index of reduced β-cell function (16). Thereafter, an annual OGTT was recommended.

PG was measured by the glucose oxidase method (the coefficient of variation was 1.4–1.5%). Insulin was measured using an Immulite immunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA). The analytical sensitivity was 2 μIU/mL.

B. Calculations of insulin secretion and sensitivity/ resistance indices derived from OGTT

Insulin secretion was estimated by two methods: (a) Early-phase insulin secretion index (IGI: Δ 0-30 insulin/Δ 0-30 glucose min) (17) and (b) corrected insulin response (CIR) [I_{30} .100/G_{30} (G_{30}-70)] (18). The trapezoidal rule was used to calculate the incremental area under the curve (AUC) for AUC- PG $_{0\text{-}120}$ and insulin (AUC-Ins $_{0\text{-}120}$).

For the determination of insulin sensitivity/ resistance the following indices were used: (a) Homeostatic Model Assessment of Insulin Resistance (HOMA- IR), (b) quantitative insulin sensitivity check index (QUICKI), and (c) Matsuda index (MI $_{0\text{-}120}$) (19-21). The latter index encompasses both hepatic and peripheral tissue insulin sensitivity. Patients were considered insulin resistant (IR) when the HOMA-IR index was > 2.5 or QUICKI < 0.4 or MI $_{0\text{-}120}$ < 4.5 (19).

Moreover, oral disposition index (oDI) was calculated by the product of IGI and insulin sensitivity (MI $_{0\text{-}120}$) (22). This index provides a measure of β-cell function adjusted for insulin sensitivity and has been shown to be predictive of development of diabetes in the general population. In our previous study of 25 β-TM patients with diabetes mellitus, the mean oDI was: 1.08 ± 0.73 vs. 4.89 ± 2.9 in β-TM patients with normal OGTT (16).

Assessment of iron overload (IOL)

IOL was evaluated at baseline and at last follow-up by serum ferritin (SF), while Magnetic Resonance Imaging (MRI) T2* of liver and heart was available only at last consultation.

SF was measured by chemiluminescence immunoassays (Beckman Access Dxl). The normal reference range values in females are 15-150 ng/mL.

The liver iron concentration (LIC) values were expressed in mg/g dry weight (d.w.) and classified into normal (LIC < 3), mild (LIC > 3 and < 7), moderate (LIC > 7 and < 14) and severe overload (LIC > 14) (23, 24). A global heart T2* value of > 20 ms was considered a conservative cut-off for insignificant myocardial IOL (25).

Statistical analysis

All numeric variables were expressed as mean ± standard deviation (SD). For the statistical analysis, a software program was used and validated, according to Alder and Roesser (26).

Comparison of variables in the two groups of patients were made using an independent sample *t* test and variables with non-normal distribution were compared using non-parametric Wilcoxons signed rank test. Pearsons correlation tests (2-tailed) were used to study correlations between variables with parametric and non- parametric distributions. Differences in proportions between the 2 groups were evaluated using the Chi-Square test and Fishers exact test. A P value < 0.05 was considered statistically significant.

Ethics

All procedures were in accordance with the 1975 Helsinki declaration and its later amendment in 2000 (http://www.wma.net/e/policy/17-c_e.). A detailed explanation of the nature and purpose of the treatment and the likely risks and benefits associated with HRT was formally given to all patients and referring centers, who were also invited to request written patients informed consent at the beginning of treatment. Ethics approval for a retrospective study was not required because patients underwent only routine diagnostic and therapeutic procedures according to current recommendations or guidelines (3,4, 11,13, 27-29). Moreover, in our retrospective study, no identifiable private patients information was collected, and an anonymized dataset was analyzed (30).

Results

A. Patients characteristics at first consultation (baseline) and HRT

The mean age at first consultation was 18.4 ± 5.8 years (range 15.6-23.6 years). All patients were regularly transfused with genotyped, white cell depleted, washed, packed red blood cells. Four of 12 patients (33.3%) with AHH had undergone splenectomy, at a mean age of 9.5 ± 1.6 years, because of increased transfusion requirements and/or the presence or signs of hypersplenism. Seven patients were on treatment with desferrioxamine mesylate (DFO: 35- 45 mg/Kg body weight, given subcutaneously via a battery-operated portable pump, over a period of 8–10 hours overnight, for 5 to 6 nights per week) and 5 with oral deferiprone (DFO: 75 mg/Kg/day, in three divided doses).

None of the patients was underweight, but one β-TM patient with SA was classified as overweight (BMI: 26.8 kg/m^2). Five patients presented with short stature (≤ 3rd centile), 1 with primary hypothyroidism and 1 with central hypothyroidism. Both the latter were on regular treatment with L-thyroxine.

AP was diagnosed in 4 patients (SF: 1908.7 ± 880.3 ng/mL), PA in 2 patients (SF:1,383 and 1,415 ng/mL), and SA in 6 patients. In SA patients, spontaneous menarche occurred at the mean age of 13.6 ± 0.6 years (SF: 1753.5 ± 1154.3 ng/mL) and SA at the age of 17.4 ± 1.6 years (SF: 910.3 ± 581.5 ng/mL), though iron load reduced significantly with efficient chelation.

The mean alanine aminotransferase (ALT) was 40.2 ± 27.9 IU/L (range 14- 104 IU/L). ALT levels were above the normal values (< 40 IU/L) in 4 out of 12 patients.

All patients were classified as level 2, meaning that "the advantages of using the HRT generally outweigh the theoretical or proven risks ". Furthermore, the treatments were based on patients preferences (31-33) and were in accordance with the ICET-A guidelines (7). Before starting HRT, the benefits and risks of treatment were discussed with the patients taking into account patients age, medical history, risk/benefit balance and personal preferences.

Six patients were treated with oral HRT in various formulations (estradiol valerate + dienogest; ethynyl

estradiol [EE] 20 +gestodene; EE 30 + gestodene; EE 20 + desogestrel). Four patients were treated with estradiol hemihydrate MX 50 patches for 21 days associated, in the last 12-14 days, with oral medroxyprogesterone acetate (MPA) at the daily dose of 5 mg, and 2 patients with patches releasing 20 μg of EE and 150 μg of norelgestromin daily (EE+NGM).

During HRT, an annual OGTT was recommended to follow the evolution of glucose homeostasis (13).

B. Glucose homeostasis at first consultation (baseline)

At baseline, normal OGTT was present in 10 (83.3%) of 12 patients, while 1 patient had impaired glucose tolerance (IGT; PG at 2-h during OGTT: 140 mg/dL), and 1 patient had isolated PG level of 165 mg/dL at 1-h during OGTT (H-NGT) (Figure 1).

In 11 patients out of 12 (91. 6%), one or more indices of IR were observed. The highest percentage of IR indices was registered for QUICKI (91.6%) followed by MI $_{0\text{-}120}$ (25.0 %) and HOMA-IR (8.3%).

The mean IGI was 1.05 ± 0.48, the CIR: 1.13 ± 0.99, and the oDI: 6.60 ± 3.45. The mean value in 8 young healthy adult controls was: IGI: 1.7 ± 1.3; CIR: 0.90 ± 0.34, and oDI: 13.6 ± 9.9 (the P value compared to β-TM patients was: 0.12; 0.53 and 0.035, respectively).

The ratio AUC $_{INS\ 0\text{-}120}$ (μU/mL)/ AUC$_{GLU\ 0\text{-}120}$ (mg/dL) was correlated with IGI (r: 0.27; P:0.38) and MI $_{0\text{-}120}$ (r: -0.74; P:0.0059). No significant statistical correlation was documented between AUC $_{INS\ 0\text{-}120}$ vs. AUC$_{GLU\ 0\text{-}120}$, and SF vs. age, ALT, PG at 0 and 2-h, AUC $_{INS\ 0\text{-}120}$ (μU/mL)/AUC$_{GLU\ 0\text{-}120}$ (mg/dL) ratio during OGTT, early-phase of insulin secretion, and

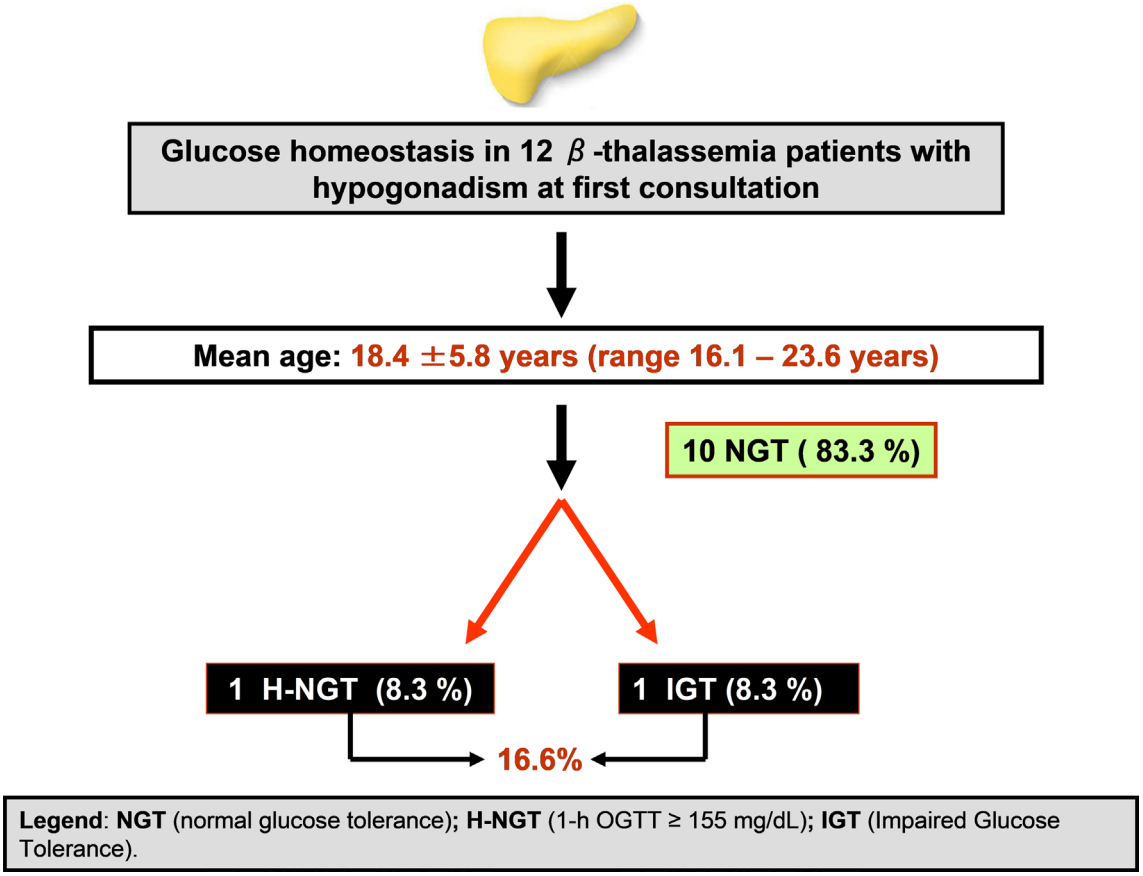

Figure 1. Number of female β-TM patients with AHH with abnormal glucose homeostasis, after OGTT, at baseline.

insulin sensitivity or resistance indices assessed during OGTT.

c. Patients characteristics at last consultation (last follow-up) versus first consultation (baseline)

The mean age and the patients clinical characteristics at last consultation are summarized in Table 1 and are compared with the data collected at baseline.

A normal OGTT was present in 7 patients (58.4 %), while 5 patients (41.6%) had abnormal glucose homeostasis (Figure 2). The first detection of dysglycemia, during the annual OGTT (assessed at 0, 60 and 120 min), was documented after 3.2 ± 1.7 years (range 1- 6 years) from the start of HRT. In these 5 patients, a fluctuation of glucose homeostasis was observed from baseline to last observation (Table 2).

In one patient on HRT with IGT at baseline (140 mg/dL at 2-h during OGTT), OGGT normalized over the 7 years of follow-up, and this was associated with a progressive decrease of SF (from 1,320 ng/mL to 374 ng/mL) on a very effective iron chelation therapy.

Five of 12 patients with glucose dysregulation presented at baseline with one or more indices of IR. The highest percentage of IR indices was observed for QUICKI (80%) followed by MI $_{0\text{-}120}$ (40 %) and HOMA-IR (20 %). Four out of 7 patients with NGT presented with one or more indices of IR: QUICKI (57.1%) and MI $_{0\text{-}120}$ (14.2%). HOMA-IR results were normal (<2.5) in all 7 patients.

During the follow-up, no significant adverse events due to HRT were documented and no additional endocrine complications were diagnosed.

Interestingly, in 3 out of 4 patients on long-term HRT with estradiol hemihydrate MX 50 patches (21 days combined with, in the last 12-14 days, oral medroxyprogesterone acetate at the daily dose of 5 mg), the PG levels, assessed during OGTT, remained stable (NGT). Their SF levels were 318; 400 and 407 ng/mL. On the contrary, the fourth patient with high SF and ALT levels (3,665 ng/mL and 63 IU/L, respectively)

Table 1. Demographic, clinical and laboratory and diagnostic data in 12 female β-TM patients with AHH on HRT at baseline and last follow-up. Data are expressed as mean ± SD.

Variables	At first consultation	At last consultation	P value
Number of β-TM patients	12	12	-
Age (yr)	18.4 ± 5.8	26.7 ± 7.9	**0.0077**
Number of splenectomized patients **Age at splenectomy (years)**	4 9.5 ± 1.6	1 additional patient (at age19.3 yrs.)	- -
Body Mass Index (kg/m2)	21.9 ± 2.4	22.0 ± 2.7	0.92
Iron chelation therapy: **Desferrioxamine (DFO) (n)** **Deferiprone (DFP) (n)** **Deferasirox (DFX) (n)** **Sequential DFP-DFO (n)**	- 7 5 0 0	- 6 2 1 3	- - - - -
Serum ferritin (ng/mL)	1745.8 ± 976.7	1008.9 ± 938.2	0.072
ALT (normal values: < 40 IU/L)	40.2 ± 27.9	20.0 ± 2.7	**0.02**
IGF-1 (ng/mL)	-	82.1 ± 47.9	-
MRI LIC (mg/g d.w.) [assessed ~ 4- 6 months before or after the last consultation]	-	4.94 ± 4.18	-
MRI global myocardial T2* (ms) [assessed ~ 4- 6 months before or after the last consultation]. Number of patients with T2*: < 20 ms (and %)	- - -	22.9 ± 8.2 - 4 (33.3%)	- - -

Legend: **AHH:** acquired hypogonadotropic hypogonadism**; n:** number; **ALT:** alanine aminotransferase**; IGF-1:** Insulin Growth Factor-1**; MRI:** Magnetic Resonance Imaging; **LIC**: liver iron concentration. In bold the P-values statistically significant.

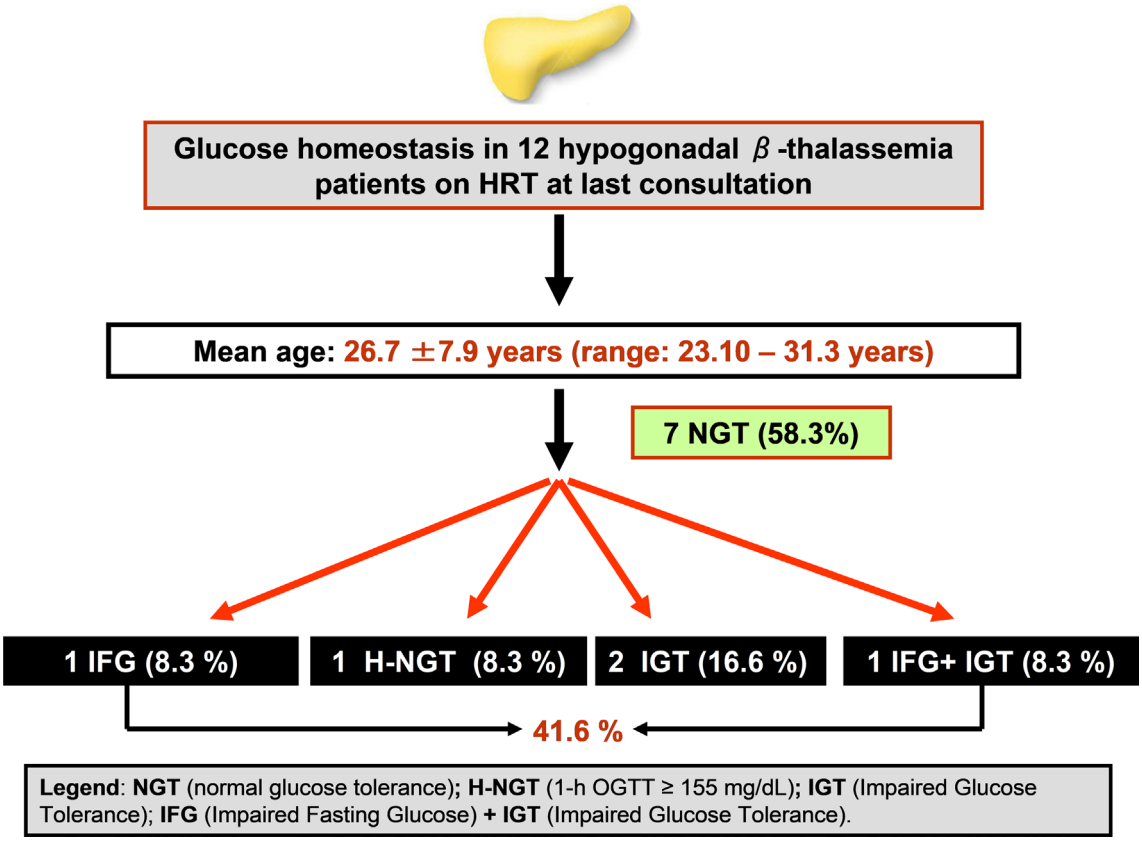

Figure 2. Number of female β-TM patients on HRT who presented, at last consultation, with normal or abnormal glucose homeostasis during OGTT.

Table 2. Long-term evolution and variability of glucose homeostasis, in 5 patients with AHH, after starting various oral HRT formulations or EE+NGM patch.

Patient (no.)	Before HRT	After 1 yr	After 2 yr	After 3 yr	After 4 yr	After 5 yr	After 6 yr	After 7 yr	After 8 yr	After 9 yr	After 10 yr
1. and SF:	NGT	NGT	NGT	NGT	**IGT 776**	NGT	NGT	**IGT**	NGT	NGT	**IGT**
2. and SF:	NGT	NGT	NGT	NGT	NGT	NGT	**IFG 741**	**IFG**	-	-	-
3. and SF:	NGT	NGT	**IFG 496**	**IFG**	NGT	NGT	**IFG**	NGT	NGT	**IGT**	**H-NGT**
4. and SF:	NGT	**IGT 1,550**	**IFG**	NGT	**IFG+ IGT**	**IFG+ IGT**	**IFG+ IGT**	**IFG+ IGT**	**IFG+ IGT**	-	-
5. and SF:	**H-NGT**	NGT	NGT	**IFG 3,156**	NGT	NGT	NGT	**IGT**	-	-	-

Legend: **SF:** serum ferritin (ng/mL) at first documentation of glucose dysregulation during follow-up; **EE+NGM** patch (releasing 20 µg of EE and 150 µg of norelgestromin); **NGT:** Normal Glucose Tolerance; **H-NGT:** 1-h OGTT ≥ 155 mg/dL; **IGT:** Impaired Glucose Tolerance); **IFG:** Impaired Fasting Glucose + **IGT:** Impaired Glucose Tolerance; **HRT:** Hormone replacement therapy in = **Patient 1:** EE 20 +norelgestromin (NGM) patch; **Patient 2**: EE 20 + desogestrel; **Patient 3:** Estradiol valerate + dienogest; **Patient 4:** Estradiol hemihydrate MX 50 patch + MPA; **Patient 5:** EE 30 + gestodene.

developed IFG + IGT. Of the remaining 4 patients with impaired glucose homeostasis, 2 were on treatment with transdermal EE+NGM patch, 1 on treatment with EE 30 + gestodene and 1 on treatment with estradiol valerate + dienogest.

Compared to results at first consultation, the PG levels during OGTT were statistically different at 0, 30, 60 and 90 minutes (Table 3 and Figure 3). However, these changes were not associated with significant differences in insulin response during OGTT (Table 3) or $AUC_{INS\ 0\text{-}120}$ /$AUC_{GLU\ 0\text{-}120}$ ratio (Insulin/Glucose AUC_{OGTT}).

Moreover, when we compared the indices of insulin secretion and sensitivity/resistance at last versus first consultation, a significant difference was observed only for CIR parameter **(**P: 0.026) (Table 4).

A significant correlation was observed between SF at PG during OGTT (at 0 min, r: 0.677; P: 0.015 and at 120 min, r: 0.7019; P: =0.0067); CIR vs. 1-h PG (r: -0.595; P:0.041); CIR vs. AUC- $INS_{0\text{-}120}$ (r: 0.613; P: 0.033), and CIR vs. IGI (r: 0.926; P:0.010).

Insulin/Glucose AUC_{OGTT} was correlated with IGI (r: 0.60; P.038), CIR (r:0.68; P: 0.013), HOMA-IR (r: 0.60; P: 0.036), QUICKI (r: -0.7488; P: 0.0051) and MI (0-120) (r: -0.84; P: 0.0005). A weak but not significant correlation was present with SF (r: 0.56; P: 0 .054). No statistically significant correlation was documented between $AUC_{INS\ 0\text{-}120}$ vs. $AUC_{GLU\ 0\text{-}120.}$

Of note, at last observation, when we analyzed the results during OGTT in 7 patients with NGT and compared the PG level, insulin response and indices of insulin secretion and sensitivity/ resistance to the first observation, an improvement of all indices of insulin sensitivity/resistance was observed (Table 5). All other parameters (IGI, CRI, PG and insulin response during OGTT, $AUC_{INS\ 0\text{-}120}$ and Insulin/Glucose AUC_{OGTT}) were not statistically different.

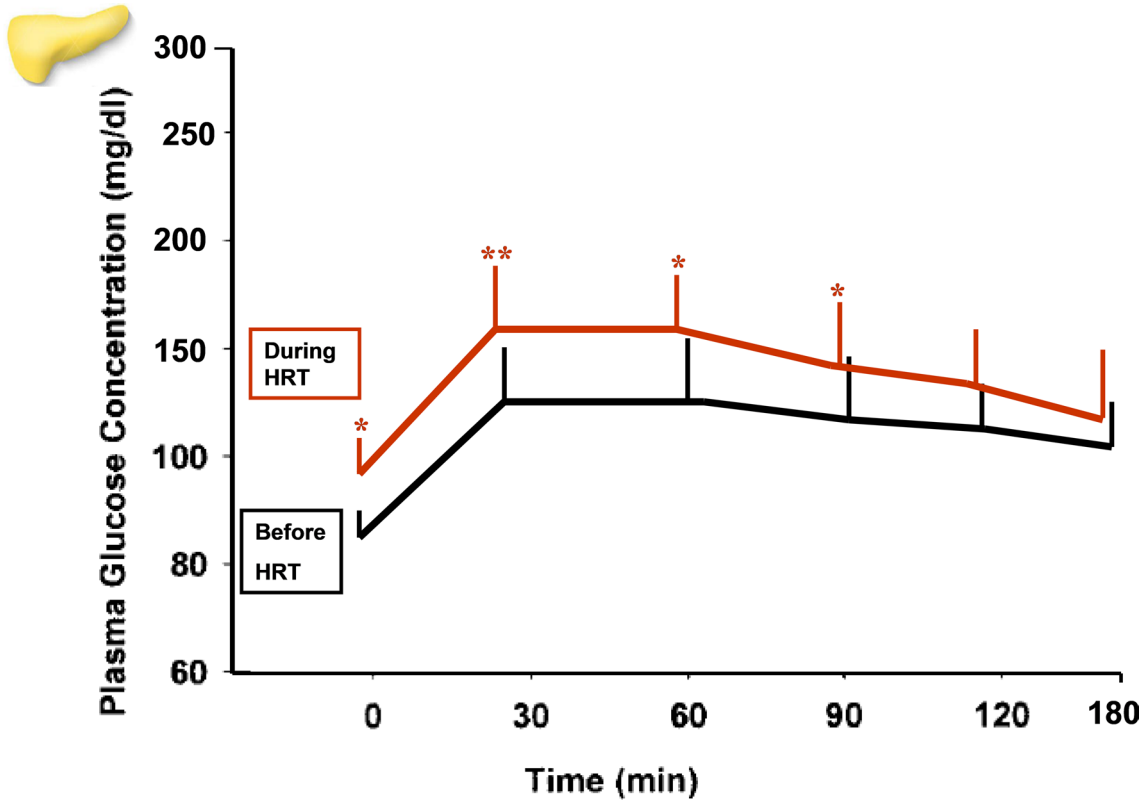

Figure 3. Profiles of plasma glucose during oral glucose tolerance test in 12 female β-TM with AHH before (at baseline) and during HRT (last consultation); (*) See table 2 for P value significance.

Table 3. Comparison of plasma glucose (PG) and plasma insulin levels during 3-h OGTT in 12 β-TM patients with AHH on HRT at first and last consultation. Data are expressed as mean ± SD.

Variables	β-TM patients with AHH (12 patients) at first consultation	β-TM patients with AHH (12 patients) at last consultation	P value
Fasting plasma glucose (PG) (mg/dL)	86.5 ± 7.4	94.8 ± 11.2	0.048
PG 30 min. after OGTT (mg/dL)	125.4 ± 19.5	151.5 ± 24.2	0.0080
PG 1-h after OGTT (mg/dL)	120.2 ± 28.3	150.3 ± 30.1	0.019
PG 2- h after OGTT (mg/dL)	110.0 ± 18.4	130.8 ± 20.7	0.016
PG 3- h after OGTT (mg/dL)	103.5 ± 16.9	107.5 ± 26.8	0.66
AUCGLU 0-120 **(mg/dL)**	229.5 ± 30.5	272.5 ± 48.7	0.016
Fasting insulin (μU/mL)	8.6 ± 2.3	6.0 ± 4.1	0.068
Insulin 30 min after OGTT (μU/mL)	53.8 ± 19.2	47.5 ± 29	0.53
Insulin 1-h after OGTT (μU/mL)	43.0 ± 28.1	41.5 ± 15.6	0.87
Insulin 2-h after OGTT (μU/mL)	26.7 ± 8.1	35.5 ± 22.0	0.20
Insulin 3-h after OGTT (μU/mL)	23.8 ±17.8	21.5 ± 18.7	0.77
AUC INS 0-120 **(μU/mL)**	74.7 ± 25.8	77.3 ± 32.9	0.83
Ratio Insulin/Glucose AUC	0.31 ± 0.09	0.28 ± 0.13	0.51

Legend: OGTT, oral glucose tolerance test; AUC $_{GLU\ 0\text{-}120}$, area under curve; AUC $_{INS}$ 0-120 , insulin area under the curve; In bold the P-values statistically significant.

Table 4. Comparison of insulin secretion and sensitivity/resistance indices during OGTT in 12 β-TM patients with AHH on HRT. Data are expressed as mean ± SD.

Variables	β-TM patients with AHH (12 patients) at first consultation	β-TM patients with AHH (12 patients) at last consultation	P value
Insulinogenic Index (IGI)	1.05 ± 0.48	0.78 ± 0.50	0.19
Corrected insulin response (CIR)	1.13 ± 0.99	0.42 ± 0.30	0.026
HOMA-IR	1.85 ± 0.60	1.47 ± 1.23	0.34
QUICKI	0.35 ± 0.02	0.37 ± 0.03	0.06
MATSUDA INDEX (MI 0-120**)**	6.70 ± 3.29	7.47 ± 2.91	0.54
Oral disposition Index (oDI)	6.60 ± 3.45	5.54 ± 2.93	0.42

Discussion

AHH in female β-TM patients is usually irreversible (34). Therefore, these patients require long-term HRT to relieve symptoms of hypogonadism and to prevent long-term health sequelae of estrogen deficiency. The type of HRT, dosage, and route of administration are extremely complex in patients with thalassemia because of the chronicity of treatment and the presence of associated comorbidities. Several publications, from various countries, demonstrated the prevalence of glucose abnormalities in β-TM patients. A questionnaire sent to 29 Centres, treating a total of 3,817 patients, reported the presence of IGT and diabetes mellitus in 6.5% and 3.2%, respectively (35).

To the best of our knowledge, no study has been conducted to investigate whether HRT has favorable or negative effects on glucose metabolism in β-TM female patients with AHH. Hence a retrospective study was promoted by ICET-A Network to assess glucose homeostasis in a group of 12 patients with β-TM treated for AHH with sex steroids, given orally or by transdermal patches.

Several interesting findings have emerged from our long-term retrospective study, in particular:

Table 5. Comparison of iron overload assessed by serum ferritin assessed, insulin secretion and sensitivity/resistance indices during OGTT in 7 β-TM patients with normal glucose tolerance (NGT) during HRT for AHH. Data are expressed as mean ± SD**.**

Variables	β-TM patients with AHH (7 patients) at first consultation	β-TM patients with AHH (7 patients) at last consultation	P value
Insulinogenic Index (IGI)	0.99 ± 0.53	1.04 ± 0.51	0.86
Corrected insulin response (CIR)	1.30 ± 1.31	0.55 ± 0.34	0.16
HOMA-IR	2.1 ± 0.55	1.08 ± 0.61	0.006
QUICKI	0.34 ± 0.013	0.38 ± 0.02	0.0008
MATSUDA INDEX (MI 0-120**)**	4.9 ± 1.0	8.20 ± 2.24	0.003
Oral disposition Index (oDI)	4.92 ± 2.89	7.71 ± 1.75	0.049
Serum ferritin (ng/mL)	1625.5 ± 1183.5	607.2 ± 353.0	0.049

a. In 9 out of 10 patients (90 %) with NGT at baseline, there were one or more indicators of IR calculated during OGTT. The gold standard test for evaluating IR is the euglycemic hyperinsulinemic clamp, but its use is limited in clinical practice due to the time involved and cost (17-22). Many studies have reported several simple methods for evaluating IR that can reduce time and cost and are relatively accurate. Compared to HOMA-IR, QUICKI seems to be a better surrogate to measure of IR in in β-TM patients.

b. The impairment of early phase insulin secretion, documented at last consultation by a significant reduction of CIR, was correlated with PG at 1-h during OGTT (r:-0.59; P: 0.041).

The 1st phase insulin secretion is primarily mediated at the level of the liver, allowing prompt inhibition of endogenous glucose production (EGP) and the 2nd phase of insulin secretion reduces EGP as in 1st phase, but to a lesser extent (36,37). It is well known that the first-phase of insulin secretion plays an important role in priming the liver and inhibiting endogenous glucose production during OGTT (37). In type 2 diabetes, the loss of the early surge of insulin release is considered a early sign that plays a pathogenic role in post-meal hyperglycemia and may require specific therapeutic intervention (38). Although the CIR parameter used to measure 1st phase insulin secretion during OGTT may be an additional valuable index for an early identification of patients at the highest risk for developing glucose dysregulation, we should not ignore that it depends on the absorption rate of glucose and neural factors (37) activated during the oral test. In addition, a maximal stimulus is not always given to the islets by 30 min. Therefore, the measures of insulin secretion derived from the OGTT are potentially subject to nonlinear relationships between glucose and insulin in subjects with different glucose tolerance.

c. The first detection of dysglycemia in patients with abnormal glucose homeostasis, at last observation, was observed at 3.2 ± 1.7 years (range 1- 6 years) after initiation of HRT. Although the effects of EE/progesterone preparations on glucose metabolism remain conflicting, a tendency towards worsening insulin sensitivity has been reported (39). Nevertheless, when we compared HOMA-IR, QUICKI and oDI before and during HRT no statistically significant differences were found.

d. Additionally, in patients with abnormal glucose homeostasis, fluctuations of glucose tolerance over 10 years follow -up was observed from 1 year to the next. This observation supports the indication for an annual evaluation of glucose tolerance, starting from the second decade of life even in patients with

regular compliance to iron chelation therapy, as observed in patients 1-3 reported in table 2. These data also are indicative of the well-known poor reproducibility of OGTT.

e. Three out of 4 β-TM patients on treatment with estradiol hemihydrate MX 50 patches plus MPA and good compliance to iron chelation therapy, documented by low SF levels, presented with stable NGT from baseline to last follow-up. This observation and the improvement of insulin sensitivity/ resistance indices documented in 7 patients with NGT and reduction of SF level at last observation (SF at baseline: 1625.5 ± 1183.5 ng/mL and at last observation: 607.2 ± 353.0 ng/mL; P= 0.049) suggest a potential positive role of SF and/or HRT on glucose homeostasis and insulin sensitivity (40). Basically, transdermal estradiol delivery systems play a more physiological role for delivering estrogen into the systemic circulation avoiding the first-pass hepatic metabolism and the exposure of supraphysiological estrogen concentrations to the liver (41).
f. Interestingly, at last follow-up, a significant direct correlation was observed between SF and PG at 0 and 120 min during OGTT. This observation confirmed the negative role of iron overload on dysfunctional insulin secretion, probably due to insufficient β cell mass and/or loss of first-phase insulin release.
g. Finally, during the follow-up, no significant adverse events due to HRT or additional endocrine complications were documented. Moreover, BMI remained stable during HRT excluding a potential negative effect of weight gain on glucose metabolism.

The limitations of our study include its retrospective nature as well as the small number of patients, that potentially decrease the accuracy of data interpretation. Moreover, the calculations of insulin secretion and sensitivity were generated by using mathematical models derived from OGTT instead of the gold-standard techniques. The strength of this retrospective study is the long-term follow-up in a group of β-TM patients on HRT, which also allowed us to evaluate the potential adverse events during HRT.

In conclusion, the reduced insulin sensitivity documented at baseline and subsequent deterioration of first-phase secretion at follow- up, highlight the evidence that reduction in both insulin sensitivity and β-cell function underlie the progression of disturbances of glucose homeostasis in β-TM patients. Our data indicate a statistically significant increase of glucose values, both fasting and at different time points after oral glucose load, over an 8-year follow-up period. The causes are not clear and may include the natural history and progression of patients or an effect of HRT or other, not well-defined, causes. Surprisingly, a large proportion of patients developed dysglycemia despite significant reduction of iron loading, as evidenced by the decrease in serum ferritin. In contrast to the proven beneficial effect of estrogens in hypogonadal females on glucose levels through reducing insulin resistance, our retrospective data suggest a possible detrimental impact of HRT on glucose handling in β-TM. Therefore, our findings need to be reproduced in larger studies of females with β-TM.

Conflict of Interest Statement: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article.

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