

**Original Article****Long-Term Follow-up of  $\beta$ -Transfusion-Dependent Thalassemia (TDT) Normoglycemic Patients with Reduced Insulin Secretion to Oral Glucose Tolerance Test (OGTT): A Pilot Study**

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**Abstract. Objective:** To study the endocrine pancreas' function in transfusion-dependent  $\beta$ -thalassemia ( $\beta$ -TDT) patients with a normal glucose tolerance test (NGT) and hypoinsulinemia. In addition, the prospective long-term follow-up using an annual oral glucose tolerance test (OGTT) to detect any abnormality of glucose metabolism.

**Patients and methods:** Seven  $\beta$ -TDT patients (mean age  $22.4 \pm 4.2$  years) with NGT and inadequate insulin response (hypoinsulinemia) to OGTT were referred for a second opinion to an Italian Centre.

**Results:** The first-phase insulin response (FPIR), expressed as the sum of 1 and 3 minutes insulin, to intravenous glucose tolerance test (IVGTT), was between the 1<sup>st</sup> and 3<sup>rd</sup> percentile in two patients and between the 3<sup>rd</sup> and 10<sup>th</sup> percentile in five. The results were not associated with  $\beta$ -cell autoimmunity. After  $43 \pm 26$  months (range 11 - 80 months) of follow-up, two patients developed impaired glucose tolerance (IGT), three both IGT and impaired fasting glucose (IFG) and two overt diabetes mellitus (DM). Interestingly, the patients who developed DM had, at baseline, the lowest value of the insulinogenic index (IGI: 0.08 and 0.25), defined as the ratio of the increment of plasma insulin to plasma glucose during the first 30 minutes after OGTT. Moreover, a significant correlation was found between the IGI at baseline and at follow-up in the patients who developed IGT with or without IFG ( $R = 0.927$ ;  $P = 0.023$ ). A significant reduction of Matsuda insulin sensitivity index (ISIM) and Insulin Secretion-Sensitivity Index-2 (ISSI-2) was documented in the study cohort at the diagnosis of IFG, IGT, and DM. There was a significant inverse correlation between ISSI-2 and area under the curve plasma glucose (AUC-PG).

**Conclusions:** These data demonstrated, for the first time, progressive deterioration in glucose homeostasis in  $\beta$ -TDT subjects with NGT and hypoinsulinemia and that the ISSI-2 index may be a valuable parameter to identify patients at high risk for developing glucose dysregulation.

**Keywords:** Transfusion-dependent  $\beta$ -thalassemia; Hypoinsulinemia; Iron overload; Oral glucose tolerance test; Intravenous glucose tolerance test; Glucose tolerance abnormalities; Insulin resistance; Follow-up.

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**Introduction.**  $\beta$ -thalassemias are amongst the commonest genetic disorders worldwide, caused by a reduction of the  $\beta$ -globin chains of the hemoglobin molecule, leading to severe chronic hemolytic anemia. Transfusion-dependent  $\beta$ -thalassemia ( $\beta$ -TDT/ $\beta$ -thalassemia major) patients present to pediatric departments in infancy and early childhood (< 3 years) with severe anemia that requires lifelong regular transfusions for survival. The disease process's culprit is the secondary iron overload from regular transfusions, which may lead to organ damage and failure, mainly involving the heart, liver, and endocrine glands. Excess iron is removed from the body by using parenteral or oral iron chelators starting from early childhood; using iron chelators regularly is difficult for many caregivers and patients, leading to poor compliance.<sup>1</sup>

In patients receiving suboptimal iron chelation, pancreatic iron loading starts in early childhood.<sup>2</sup> The pathogenesis of glycemic abnormalities in  $\beta$ -TDT patients is complex and multifactorial, and significantly different from the pathogenesis of glucose metabolism's dysregulation in a normal individual, particularly children and adolescents. Several studies have shown that insulin resistance and insulin deficiency mark both the prediabetic state and diabetes in thalassemia.<sup>3-5</sup> Insulin secretory defects, however, may originate from pancreatic  $\beta$ -cell damage rather than from insulin resistance.<sup>6</sup>

To evaluate the possible role of autoimmunity in the pathogenesis of diabetes, Monge et al.<sup>7</sup> studied a cohort of 53  $\beta$ -TDT patients, including twelve patients with diabetes (22.6%). To be evaluated about the activation of an autoimmune response, individuals were tested for islet cell antibodies (ICA), glutamic acid decarboxylase (GAD) autoantibodies, insulin autoantibodies (IAA), and serum antinuclear antibodies (ANA). The study demonstrated evidence of immune system activation against pancreatic  $\beta$ -cells in  $\beta$ -TDT patients. The authors suggested that iron deposition may act through oxidative damage as an environmental factor that triggers the autoimmune response.<sup>7</sup>

Overall, these diabetogenic factors have a cumulative effect that seems to be progressive and can lead to glucose intolerance in long-term.<sup>8</sup>

In the light of these observations, we report seven  $\beta$ -TDT patients with normal glucose tolerance test (NGT) and reduced insulin response (hypoinsulinemia), after an oral glucose tolerance test (OGTT), referred for a second

opinion, from Italian Centers taking care of patients with hemoglobinopathies. Due to very uncommon observations, their long-term natural history, assessed by an annual OGTT, is reported with the aim of detecting any abnormality of glucose metabolism.

### **Patients and Methods.**

*At baseline.* Our cross-sectional study started in 2008. Seven Italian Centers, taking care of patients with hemoglobinopathies, referred for a second opinion to an ICET-A (International Network of Clinicians for Endocrinopathies in Thalassemia and Adolescence Medicine) Italian Center, seven  $\beta$ -TDT patients (mean age  $22.4 \pm 4.2$  years) with NGT associated to hypoinsulinemia during OGTT. The long-term patients' natural history of OGTT is also reported. According to Crofts et al.,<sup>9</sup> the insulin secretory capacity was defined as reduced if all insulin values during OGTT were  $\leq 30$   $\mu$ U/mL. Based upon plasma glucose results on OGTT, patients were classified according to the American Diabetes Association (ADA) criteria.<sup>10</sup>

The following data were collected from each subject: demographic data, age at first transfusion, the interval between transfusions, type and compliance to iron chelation therapy, anthropometric data [weight, height, body mass index (BMI)], pubertal status, and associated endocrine complications. BMI was calculated as body weight in kilograms divided by height in meters squared.

A subject was considered obese when BMI exceeded 30  $\text{Kg}/\text{m}^2$ , overweight when BMI was 25 - 30  $\text{kg}/\text{m}^2$ , of average weight when BMI was 18.5-25  $\text{kg}/\text{m}^2$ , and underweight when the BMI was < 18.5  $\text{kg}/\text{m}^2$ .

*First step: Study of autoimmunity and first-phase insulin release (FPIR) after intravenous glucose tolerance test (IVGTT).* The  $\beta$ -cell autoimmunity was assessed in all patients by GAD, ICA, and IAA. The samples were analyzed for ICA by immunofluorescence and for GAD and IAA by specific radioligand binding assays. Waiting for autoimmunity results against pancreatic  $\beta$ -cells, an intravenous glucose tolerance test (IVGTT) was performed between 08.30 and 09.30, after fasting for 8-10 hours. Patients consumed a regular diet for 3 days and avoided smoking before the test.

An intravenous catheter was placed in the antecubital vein, and an intravenous bolus of 0.5 g glucose/kg body weight (maximum 35 g, as 25% water solution) was injected manually through an indwelling intravenous

cannula in the contralateral antecubital vein over 3 - 4 minutes. The end of glucose infusion was defined as time zero. Blood was assessed for insulin radioimmunoassay at baseline (completion of glucose infusion) and after 1, 3, 5, and 10 minutes. The sum of insulin values at 1 and 3 minutes was used as an index of the first phase insulin response (FPIR).<sup>11,12</sup> For FPIR, the 1<sup>st</sup> percentile is 48  $\mu$ U/ml, 3<sup>rd</sup> percentile 56  $\mu$ U/ml, 5<sup>th</sup> percentile 64  $\mu$ U/ml, 10<sup>th</sup> percentile 81  $\mu$ U/ml, and 50<sup>th</sup> percentile 162  $\mu$ U/ml.<sup>12</sup>

*Other laboratory assays and cardiac imaging.* The serum alanine aminotransferase (ALT) level was determined by an automated analyzer (normal range 0–40 mU/L). Serum ferritin was measured by immunoassays. The 90<sup>th</sup> percentile of reported average values is 201–243: ng/mL.<sup>13</sup>

Plasma glucose was measured using an automated glucose oxidase reaction (Glucose Analyser, Ames). Plasma samples were centrifuged at 4°C, separated, and stored at –20°C until assay. Plasma insulin was determined by a commercial solid-phase radioimmunoassay technique (Coat-A-Count insulin kit, Diagnostic Products Corporation, Los Angeles, CA) with intra- and inter-assay coefficients of variance of 3.3% and 2.5%, respectively.

Cardiac T2\* was assessed by magnetic resonance imaging (MRI) using a 1.5 T scanner. A conservative cut-off value of heart T2\* > 20 ms was considered normal.<sup>14</sup>

*Follow-up.* After IVGTTs, all patients were followed yearly with an OGTT to detect any potential glucose abnormalities, including quantitative and qualitative defects in insulin secretion, using the following methods and calculation indices;

*Plasma glucose, insulin, and islet  $\beta$ -cell function indices from the OGTT.* One week following the blood transfusion, a 75-g OGTT was performed in the morning after an overnight fast. Blood samples were collected from the venous catheter at 0, 30, 60, 90, 120, and 180 minutes for plasma glucose and insulin assessment. Participants remained seated for the entire testing period.

Based upon plasma glucose results on OGTT, patients were classified according to the American Diabetes Association (ADA) criteria<sup>10</sup> into the following categories:

- Normoglycemia: FPG < 100 mg/dL and 2-h PG < 140 mg/dL;
- IFG: FPG between 100 and 125 mg/dL;
- IGT: 2-h PG between 140 and 199 mg/dL;
- DM: Fasting plasma glucose (FPG)  $\geq$  126 mg/dl or 2-hour plasma glucose (2-h PG)  $\geq$  200 mg/dL.

Different indirect indices were applied for insulin resistance and sensitivity recognition; among them those

calculated from fasting glucose and insulin concentration and those derived during the OGTT, including Insulinogenic Index (IGI), plasma glucose (PG), and insulin (INS) area under the curves (AUC- PG 0–120 min and AUC-INS 0–120 min), Homeostasis Model Assessment of Insulin Resistance (HOMA1-IR), Quantitative Insulin sensitivity Check Index (QUICKI), Matsuda insulin sensitivity index (ISIM), and Insulin Secretion-Sensitivity Index-2 (ISSI-2). AUC- PG, and AUC-INS during OGTT were calculated with the trapezoid method.<sup>15-19</sup>

*Statistical analysis.* Data are presented as means  $\pm$  standard deviation (SD). Statistical comparison between parameters was made using the paired “t” test. Simple linear regression tested the correlations between variables. For the statistical analysis, a software program was used and validated, according to Alder and Roesser.<sup>20</sup> A p-value < 0.05 was considered statistically significant.

*Ethics.* All procedures were in accordance with the 1964 Helsinki declaration and its later amendments. According to the Italian regulations, the local Ethics Committee's approval was not required for the following reasons: no identifiable private information was collected; patients underwent only routine diagnostic and therapeutic procedures according to current guidelines;<sup>10</sup> an anonymized dataset was analyzed. Informed consent was obtained from all patients after a detailed explanation of the study's nature and purpose and the likely risks and benefits of study participation.

## Results.

*Clinical and laboratory characteristics.* The patients' demographic data and other parameters are shown in **Table 1**. All patients were on regular blood transfusions and iron chelation therapy. They were neither overweight/obese nor underweight.

Five of seven patients were on desferrioxamine (DFO) monotherapy with an average daily dose of 40  $\pm$  5.34 mg/kg body weight (range: 35- 50 mg/kg body weight), given subcutaneously by pump for 7 to 8 hours per night, for 5 to 6 days a week. Two patients were on oral deferiprone (DFO) monotherapy (total dose 75 mg/kg/day, divided into 3 doses). At baseline, their mean serum ferritin levels were 1,562.5  $\pm$  834.7 ng/mL (range: 955-3,015 ng/mL) (**Table 1**).

The highest mean serum ferritin level ("peak level") registered in the course of previous years was 3,664.2  $\pm$  1,567.5 ng/mL (range: 6,000-2,250 ng/mL). ALT was elevated in 3 patients (patients 2, 5, and 7 with DFO). Cardiac T2\* was impaired in one patient (18.8 msec) with a peak ferritin level of 5,400 ng/mL.

**Table 1.** Clinical and laboratory characteristics of 7 patients with  $\beta$  - TDT at baseline.

Patient no./Sex	Age (years)	BMI (Kg/m <sup>2</sup> )	AEC	ALT (mU/mL)/ HCV antibodies	SF peak (ng/ml)	SF at OGTT (ng/ml)	Iron chelation therapy at OGTT	Global Cardiac T2* (msec)
1/ F	29.1	21.8	SA-SCH	33 positive	6,600	1,055	DFO	NA
2/ F	20.9	21.6	SA	62 negative	2,250	1,158	DFO	40
3/ M	24.0	21.3	None	41 negative	2,520	955	DFP	NA
4/ M	20.7	19.9	HH	19 negative	5,400	1,195	DFO	18.8
5/ F	27.1	19.5	SA	65 negative	2,320	3,015	DFO	NA
6/ M	15.5	19.7	None	29 negative	3,560	845	DFO	28.8
7/M	20.0	22.0	CH	86 negative	3,000	2,715	DFP	31.7
<b>Mean <math>\pm</math> SD</b>	<b>22.4 <math>\pm</math> 4.2</b>	<b>20.8 <math>\pm</math> 1.0</b>	=	<b>42.7 <math>\pm</math> 21.6</b>	<b>3,664.2 <math>\pm</math> 1,567.5</b>	<b>1,562.5 <math>\pm</math> 834.7</b>	<b>5 DFO 2 DFP</b>	=

**Legend:** BMI = Body mass index; AEC: associated endocrine complications; ALT: serum alanine aminotransferase; NA: not available; SA: secondary amenorrhea; SCH: Subclinical hypothyroidism; HH: hypogonadotropic hypogonadism; CH: Central hypothyroidism; ALT: alanine aminotransferase: normal values: <40 mU/ml; SF: serum ferritin, 50<sup>th</sup> centile: 56-105 ng/mL in males and 27-35 ng/mL in females; OGTT: Oral glucose tolerance test; DFO: desferrioxamine; DFP: deferiprone.

*Glucose tolerance at baseline.* At baseline, all patients had normal glucose values with reduced insulin secretion on OGTT. The peak of plasma glucose and insulin response, after OGTT, was delayed (60'-90' minutes) in 3 patients (Table 2).

No statistical correlation was observed between age and BMI, AUC-PG, AUC-Ins, and peak of serum ferritin level. Similarly, a non-significant correlation was found between HOMA-IR, QUICKI, and ISSI-2 with AUC-PG and AUC-INS. Conversely, a significant correlation was found between ISIM and AUC-INS (R= 0.7824; P: 0.037) but not with AUC-PG. An inverse correlation was present between HOMA-IR and QUICKI (R= -0.9567; P: 0.0007).

Correlations between serum ferritin levels (peak over previous years ) with HOMA-IR, QUIKI, AUC-PG, and AUC-INS were not significant. An inverse correlation was present between serum ferritin at the time of study vs. ISIM and ISSI-2 (R= -0.962; P: 0.00053 and R= -0.8459; P: 0.016, respectively).

*Adverse effects of OGTT.* There were no adverse events secondary to OGTT, and none of the subjects experienced hypoglycemia.

*FPIR after IVGTT.* In two patients, the FPIR value (expressed as the sum of 1 + 3 min insulin) was between

**Table 2.** Oral glucose tolerance test (OGTT) and first-phase insulin response (FPIR), after IVGTT, at baseline in seven  $\beta$ - TDT patients.

Patient no. /Sex	Age (yrs)	0' PG/INS	30' PG/INS	60' PG/INS	90' PG/INS	120' PG/INS	180' PG/INS	AUC-PG and AUC-INS (0-120)	IGI	FPIR (1+3 min.)
1/F	27.1	94 4	100 15	117 19	94 9	105 9	76 5	218.0 28.25	1.83	53
2/F	20.9	76 3	110 19	135 21	115 11	123 13	111 6	255.7 33.25	0.56	79
3/M	24.0	80 7	109 22	103 11	114 18	97 11	60 4	173.0 28.25	0.51	82
4/M	20.7	88 7	118 20	112 15	124 17	106 15	94 8	246.0 32.25	0.43	58
5/F	27.1	97 9	171 15	124 12	91 12	110 14	91 7	282.0 28.0	0.08	66
6/M	15.5	81 5	124 16	124 18	118 12	103 11	106 5	247.0 29.5	0.25	52
7/M	20.0	99 2	131 29	137 29	100 8	121 24	87 11	278.7 45.0	0.84	72

**Legend = PG:** plasma glucose (mg/dL); **INS:** insulin ( $\mu$ U/mL); **AUC-PG and AUC-Ins:** PG-Ins (0)+PG-Ins (30) x2 +PG-Ins (60)x3+PG-Ins (120) x 2/4; **Normal insulin values ( $\mu$ U/mL) before and during OGTT= 0':7  $\pm$  3; 30': 46.2  $\pm$  25.3; 60': 37  $\pm$  17.6; 90': 35.2  $\pm$  12.2; 120': 24.1  $\pm$  12.2; 180':10.7  $\pm$  7.7; **Insulin area :** 92.9  $\pm$  44.8 (From: De Sanctis et al. Postgrad Med J.1985; 61: 963-967); **IGI:** Insulinogenic Index.**



**Table 3.** Age at first documentation of glucose homeostasis impairment in 7  $\beta$ -TDT patients followed yearly with an OGTT.

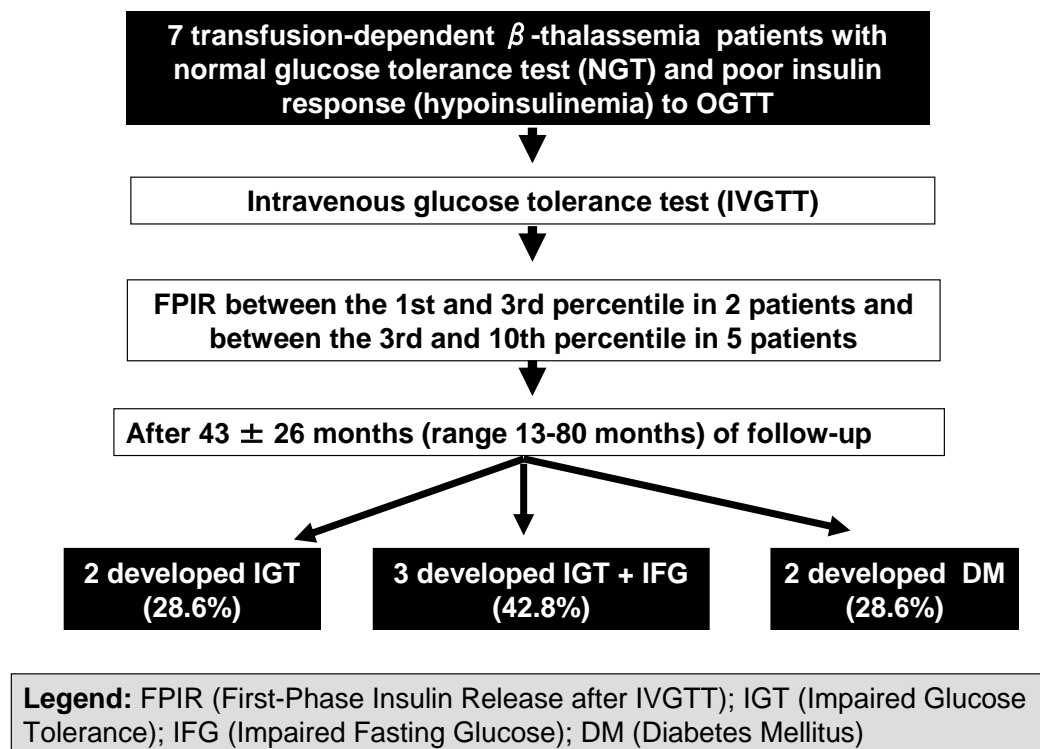
Patient no/Sex	Age (yrs)	Time interval (yrs) after baseline	0' PG/INS	30' PG/INS	60' PG/INS	90' PG/INS	120' PG/INS	180' PG/INS	AUC-PG and AUC-INS (0-120)	IGI	SF/ALT
1/F	29.1	2 yrs IFG+IGT	122 6	112 14	125 23	162 22	147 21	112 23	284.2 37.7	0	1,100 33
2/F	27.9	7 yrs IFG+IGT	103 7	190 38	148 20	169 27	140 17	105 8	327.5 46.0	0.35	859 63
3/M	30.6	6.6 yrs IFG+IGT	102 5.6	122 11	162 27	161 24	145 19	85 5.6	296.0 39.5	0.27	1,475 27
4/M	22.8	2.1 yrs IGT	91 5	104 5	129 14	132 15	157 13	125 13	272.7 33.3	0	1,480 32
5/F	30.4	3.3 yrs DM	102 8	212 10	207 11	209 13	206 12	140 12	415.2 23.2	0	2,600 37
6/M	16.6	1.1 yrs DM	138 3	170 9	197 16	223 11	220 11	182 12	411.7 23.5	0.18	1,340 29
7/M	23.1	3.1 yrs IGT	85 8	188 21	158 23	131 25	140 15	119 13	325.0 39.2	0.12	536 70

**Legend = IFG:** Impaired fasting glucose; **IGT:** Impaired glucose tolerance; **DM:** Diabetes mellitus; **PG:** plasma glucose (mg/dL); **I:** insulin ( $\mu$ U/mL); **AUC-PG and AUC-Ins:** PG-Ins (0)+PG-Ins (30) x2 +PG-Ins (60)x3+PG-Ins (120) x 2/4; **Normal insulin values ( $\mu$ U/mL) before and during OGTT= 0':  $7 \pm 3$ ; 30':  $46.2 \pm 25.3$ ; 60':  $37 \pm 17.6$ ; 90':  $35.2 \pm 12.2$ ; 120':  $24.1 \pm 12.2$ ; 180':  $10.7 \pm 7.7$ ; **Insulin area :**  $92.9 \pm 44.8$  (From: De Sanctis et al. Postgrad Med J. 1985; 61: 963-967); **IGI:** Insulinogenic Index.**

the 1<sup>st</sup> and 3<sup>rd</sup> percentile (patients 1 and 6), and in five patients between the 3<sup>rd</sup> and 10<sup>th</sup> percentile (**Table 2**). No correlation was observed between FPIR and age, AUC-PG, AUC-Ins, the peak of serum ferritin level, or serum ferritin at baseline. These results were not associated with  $\beta$ -cell autoimmunity.

*Glucose tolerance, insulin and islet  $\beta$ -cell function indices from the OGTT during annual follow-up.* During the annual OGTT follow-up, glucose homeostasis's first

alteration was documented after  $43 \pm 26$  months (range 13-80). Two patients developed IGT (patients 4 and 7), three IFG associated with IGT (patients 1, 2 and 3), and two overt DM (patients 5 and 6) within 3.3 and 1.1 yrs, respectively (**Table 3**). All seven patients had abnormal 2-hour plasma glucose (**Figure 1**). A strong linear correlation was also observed between the FPIR value at baseline and interval (expressed in months) of the appearance of glucose homeostasis abnormalities ( $R=0.8753$ ,  $P: 0.009$ ).



**Figure 1.** Outcome of 7  $\beta$ -TDT patients with normal OGTT and hypoinsulinemia followed for 13-80 months.

**Table 4.** Follow-up of indirect indices of insulin secretion and resistance at baseline and at the diagnosis of IGT and DM in patients with  $\beta$ -TDT.

HOMA 1-IR- Before	HOMA 1-IR- After	QUICKI Before	QUICKI After	ISIM Before	ISIM After	ISSI-2 Before	ISSI-2 After
1.14±0.58	1.54±0.37	0.38±0.03	0.34±0.02	12.35 ± 2.80	8.57 ± 2.42	204.7 ± 66.8	112.2 ± 34.5
<b>P = 0.16</b>		<b>P = 0.17</b>		<b>P = 0.009</b>		<b>P = 0.003</b>	

**Legend = Normal values of Homeostasis Model Assessment of Insulin Resistance (HOMA1-IR):** 2.31 (2.21–2.46); **Quantitative Insulin Sensitive Check (QUICKI):** 0.34 (0.33–0.34); **Matsuda insulin sensitivity index (ISIM):** 6.00 (5.06–6.12); **Insulin secretion-sensitivity index-2 (ISSI-2):** 304 (290–327). (From: Płaczkowska et al. Ann Agric Environ Med. 2020;27:248-254).

The AUC-Ins120/AUC-PG 120 was not statistically different between baseline values vs. last observation (P: 0.16). However, the  $\beta$ -TDT patients who developed DM had the lowest IGI index values at baseline (0.08 and 0.25). Throughout the entire follow-up, five patients developed IGT; there was a significant difference in baseline IGI in these patients compared to those who did not develop IGT (R= 0.927; P: 0.023), independently from IFG presence.

There was no significant change in HOMA-IR and QUICKI during the follow-up. On the other hand, a significant reduction in the ISIM and ISSI-2 was documented in the whole study cohort at IFG-IGT and DM diagnosis. There was a significant inverse correlation between ISSI- 2 and AUC-PG (R= -0.9617, P: 0.0005) (**Table 4**).

No significant BMI changes occurred in patients from the baseline to the end of the follow-up period (20.8 ±1.0 vs. 21.1 ± 1.2 Kg/m<sup>2</sup>; P: NS). The mean serum ferritin level did not differ at baseline or at the occurrence of IGT/IFG/DM (1,562 ± 901.6 vs. 1,341 ± 653.3 ng/mL; P: NS).

Due to the limited number of  $\beta$ -TDT patients, a comparison of collected parameters between males and females was impossible.

**Discussion.** Disturbances of glucose tolerance occur in a significant number of adolescent and adult patients with  $\beta$ -TDT who are at high risk for developing abnormal glucose handlings, such as IFG, IGT, and DM. These glucose disturbances are mainly due to decreased insulin secretion and insulin resistance (IR) secondary to iron overload.<sup>1-6</sup>

OGTT is widely used in clinical settings to diagnose patients with IGT and DM, based on ADA recommendations.<sup>10</sup> OGTT is a valuable tool in diabetes research and is commonly used for screening, evaluating disease progression, monitoring treatment, and thoroughly studying physiological and pathophysiological conditions.<sup>21</sup> Because of the increased risk of glucose disturbances in  $\beta$ -TDT patients with iron overload, there are recommendations for annual evaluation of glycemic status in children over ten years.<sup>22</sup>

However, OGTT gives only a crude estimate of  $\beta$ -cell secretory function. The initial insult is considered insulin

resistance, which, as long as it is compensated by hyperinsulinemia, does not lead to hyperglycemia. Finally, when pancreatic  $\beta$ -cells are unable to secrete an increased amount of insulin, to compensate for insulin resistance, hyperglycemia develops.<sup>3-6</sup>

In this study, patients with  $\beta$ -TDT, despite exhibiting normal glucose tolerance as defined by standard glucose homeostasis criteria, presented with impaired insulin secretion (hypoinsulinemia) during OGTT. The reduced insulin response to OGTT was further confirmed by FPIR values (between the 1<sup>st</sup> and 3<sup>rd</sup> percentile in two patients and in five between the 3<sup>rd</sup> and 10<sup>th</sup> percentile). The potential role of autoimmunity was excluded by the assessment of islet cell antibodies (ICA), glutamic acid decarboxylase (GAD) autoantibodies, and insulin autoantibodies (IAA).

Although we cannot identify the cause of the low insulin levels found during OGTT at baseline, we can speculate that these patients had a higher insulin sensitivity. However, we cannot exclude, on the basis of long-term follow-up, the decreased  $\beta$ -cell secretory capacity was due to an initial and progressive impairment of pancreatic  $\beta$ -cells toxicity of iron overload,<sup>6</sup> and/or to loss of pancreatic micro-vascularity.<sup>23-25</sup>

A strong linear correlation was observed between the FPIR value at baseline and the time interval of the appearance of glucose abnormalities (R= 0.8753, P: 0.009). In two patients, the FPIR value (expressed as the sum of 1 + 3 min insulin) was between the 1<sup>st</sup> and 3<sup>rd</sup> percentile (patients 1 and 6), and in five, between the 3<sup>rd</sup> and 10<sup>th</sup> centile. Nevertheless, the insulin response to IVGTT in all patients was higher compared to OGTT. In healthy individuals, the incretin effect accounts for 70% of the insulin response after oral glucose administration. However, we can speculate a possible reduction of the incretin effect in pivotal experiments; to assess the incretin effects the comparison between oral and i.v. glucose stimuli were performed using well-matched glucose concentrations. That was not the case in our subjects.

From the baseline, all patients were followed yearly with an OGTT. After a total period of 43 ± 26 months (range: 11-80) of follow-up, two patients developed IGT, three both IFG and IGT, and two developed DM. Nevertheless, no statistical difference was found

between the AUC-Ins120/ AUC-PG120, considered an index of total insulin secretion during the OGTT. This observation suggests that a decrease in insulin sensitivity would be a factor involved in the deterioration of glucose tolerance when the insulin secretion is compromised. In patients with  $\beta$ -TDT, IR may be due to: (a) a direct effect of iron overload to pancreatic  $\beta$ -cells and/or (b) hepatic dysfunction leading to reduced hepatic clearance of insulin resulting in impaired glucose homeostasis.<sup>26-27</sup> Moreover, IR can be related to the variation of qualitative and quantitative nutritional intake and low physical activity.

Several techniques have been used in humans to assess IR. Methods for quantifying  $\beta$ -cell sensitivity to glucose (hyperglycemic clamp technique) and tissue sensitivity to insulin (euglycemic insulin clamp technique) are generally recognized as the gold standard reference for assessing IR. However, these methods are laborious, expensive, and inconvenient to patients or study subjects and are not routinely available to every physician. Since OGTT is cheap and straightforward, many mathematical models' formulas have been developed using OGTT parameters. We calculated the most widely used surrogate indices: IGI, HOMA-IR, QUICKI, ISIM, and ISSI-2, calculated from fasting glucose and insulin concentration, and those derived OGTT evaluation.<sup>28-30</sup>

In brief, IGI is calculated as the ratio of plasma insulin's increment to glucose concentration 30 min after an OGTT. The loss of this early insulin release is a feature of the prediabetic condition. HOMA and QUICKI are mathematically related and provide essentially identical information. Both primarily reflect hepatic insulin resistance rather than peripheral insulin resistance.<sup>31</sup>

ISIM combines two terms that account for insulin sensitivity of the hepatic as well as the peripheral tissues. One part of the equation consists of a hyperbolic conversion of the product of fasting plasma glucose and insulin as a measure of hepatic sensitivity. The second accounts for whole-body insulin sensitivity, described by the inverse product of the mean glucose and insulin concentration after the glucose load. ISSI-2 is defined as the ratio of the area under the insulin curve to the area under the glucose curve, multiplied by the Matsuda index; it constitutes a surrogate measure of insulin secretion relative to insulin sensitivity and emphasizes the pivotal role of impaired insulin secretion in the development of dysregulation of glucose homeostasis. Substantially, it refers to the relationship between insulin sensitivity and insulin secretion.<sup>15-19</sup>

Our results confirm that the ISSI-2 index may be valuable parameters to identify  $\beta$ -TDT patients at the highest risk for developing glucose dysregulation.<sup>32</sup>

Interestingly, the  $\beta$ -TDT patients who developed DM had, at baseline, the lowest values of the IGI index (0.08

and 0.25). Moreover, a significant correlation was found between IGI at baseline in those patients who developed IGT compared to those who did not, regardless of IFG ( $R= 0.927$ ;  $P: 0.023416$ ). No significant changes in HOMA-IR and QUICKI were observed during follow-up. Conversely, a significant reduction of ISIM and ISSI-2 was documented in the whole study cohort at the diagnosis of IFG-IGT and DM, with a significant inverse correlation between ISSI-2 and AUC-PG. Therefore, ISIM and ISSI-2, which include post-load glucose and insulin concentrations, provided a more accurate estimate of whole-body insulin sensitivity than HOMA-IR or QUICKI, derived from fasting measurements only, thus constituting a more sensitive tool for detecting alterations of glucose sensitivity/resistance in  $\beta$ -TDT patients.

Our study has several limitations: a) the small number of patients enrolled in the study, but this seems inevitable as the profile of glucose dysregulation in TDT is reported for the first time and is very rare; b) the use of surrogate indices for assessing insulin sensitivity, and c) the evaluation of iron overload assessed with serum ferritin not associated to the evaluation of pancreatic iron stores by magnetic resonance imaging (MRI). However, the major strength is the long-term follow-up study of an uncommon group of  $\beta$ -TDT patients with NGT and hypoinsulinemia who developed, after  $43 \pm 26$  months, a glucose dysregulation.

**Conclusions.** There are limited data, if any, about the function of the endocrine pancreas in normoglycemic  $\beta$ -TDT patients with impaired insulin secretion on OGTT. In our experience, IVGTT, a test that nowadays is not routinely included in the screening of glucose metabolism disturbances in  $\beta$ -TDT patients, could be useful in selected patients with NGT and reduced insulin response (hypoinsulinemia) after OGTT. The longitudinal evaluation of surrogate measures of insulin secretion and/or sensitivity allowed us to demonstrate that a weakening of peripheral insulin action may contribute to the development of glucose homeostasis impairment. However, a larger number of patients is necessary to understand better the respective roles of the progressive reduction of insulin secretion and the variation of insulin sensitivity. Our observations also support the need for a continuous follow-up with regular OGTT and timed counseling to promote lifestyle changes in high-risk subjects. As demonstrated in the general population and patients with type 2 diabetes, physical activity programs, diet changes, and pharmacological interventions could be useful measures to improve glucose tolerance in patients with  $\beta$ -TDT. Further studies are required to determine the possible benefits of insulin oral secretagogues and to establish the best treatment for this patient group.

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