Serum Insl3 As A Biomarker of Hypogonadism in Beta-Thalassemia Major

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Abstract: Objective- Beta-thalassemia major (BTM) frequently results in hypogonadism, primarily due to iron overload and oxidative stress. Insulin-like growth factor-3 (INSL3), a hormone secreted by Leydig cells, may serve as a potential biomarker for the early detection of hypogonadism. This study aimed to explore the relationship between serum INSL3 levels and hypogonadism in a cohort of patients with Beta-thalassemia major.The study included 111 participants with Beta-thalassemia major, divided into three groups: 30 individuals with hypogonadism (Group I), 51 without hypogonadism (Group II), and 30 healthy controls (Group III). Hormonal and biochemical assays were performed. INSL3 was quantified using a commercial ELISA kit, while levels of testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and inhibin B were measured using chemiluminescent immunoassays on automated analyzers. Ferritin, iron, unsaturated iron-binding capacity (UIBC), and total iron-binding capacity (TIBC) were assessed using colorimetric methods on an automated biochemical analyzer. Malondialdehyde (MDA) levels were measured through the thiobarbituric acid reactive substances (TBARS) assay, and Ischemia-Modified Albumin (IMA) was determined with the albumin cobalt binding (ACB) test.Serum INSL3 levels were significantly lower in Group I compared to Groups II and III. Additionally, a notable positive correlation was observed between INSL3 and inhibin B. Multiple regression analysis identified INSL3 as an independent predictor of hypogonadism. Receiver Operating Characteristic (ROC) analysis for INSL3 demonstrated an area under the curve (AUC) of 0.893, indicating excellent diagnostic accuracy.In patients with Beta-thalassemia major, INSL3 levels are diminished in the presence of hypogonadism, alongside elevated markers of oxidative stress and increased iron overload. This finding highlights the potential connection between INSL3 and male hypogonadism in the context of BTM.

1 INTRODUCTION

Beta-thalassemia major (BTM) is a group of recessively inherited autosomal disorders of hemoglobin synthesis, resulting from mutations in the beta-globin gene that lead to varying degrees of defective beta-chain production, an imbalance in alpha/beta-globin chain synthesis, ineffective erythropoiesis, and anemia. Improved survival in thalassemic patients has led to the emergence of previously unrecognized complications, such as Hypogonadism [1]. Hypogonadism is one of the most frequent complications in transfusion-dependent thalassemia patients. Iron overload is a key reason behind Hypogonadism in thalassemia patients. Iron will be deposited as hemosiderin and ferritin in various body organs, mainly the myocardium, liver, spleen, and endocrine organs. The deposited iron led to the formation of Reactive Oxygen Species (ROS) such assuperoxide anion (O^{2-}), hydroxyl radical (OH), single oxygen and hydrogen peroxide (H2O2), that responsible for development of oxidative stress in thalassemia major patients via Fenton reaction [2], [3]. One of the markers of oxidative stress is malondialdehyde (MDA), which is often used as an indicator of lipid peroxidation [4]. The levels of MDA in the blood of beta-thalassemia patients reflect the iron burden and systemic damage, and the readings are often high. Another key biomarker is ischemia-modified albumin (IMA), a modified form of serum albumin that forms under conditions of oxidative stress. Oxidative stress, redox-active forms of iron, and generation of ROS are well documented in patients with β-thalassemia major. It is therefore possible that under such conditions, the structure of human serum albumin is modified in a way that

permits excessive production of IMA [5]. Insulin-like growth factor 3 peptide (INSL3) is a member of the insulin-like peptide superfamily and is the only known physiological ligand of relaxin family peptide receptor 2 (RXFP2), a G protein-coupled receptor (GPCR). In mammals, INSL3 is primarily produced in both testicular Leydig cells and ovarian theca cells; however, circulating levels of the hormone are much higher in males than in females. The INSL3/RXFP2 system plays a crucial role in the development of the gubernaculum, facilitating the initial transabdominal descent of the testis, and in maintaining proper reproductive health in men. Studies by Ivell and Anand-Ivell (2023) and Adamczewska et al. (2022) have highlighted its diagnostic potential in primary testicular dysfunction [6], [7]. However, INSL3 has not been extensively studied in the context of chronic conditions such as beta-thalassemia major, where functional Hypogonadism may precede hormonal. The novelty of our study lies in evaluating the role of INSL3 in the early detection of Hypogonadism in male beta-thalassemia major patients. To our knowledge, this is the first comprehensive study examining the interplay between INSL3, Hypogonadism, and traditional hormones in the context of males with beta-thalassemia major. The aim in this study is to investigate the relation between serum INSL3 levels and hypogonadism in a sample of Iraqi BTM.

2 MANUSCRIPT PREPARATION

2.1 Patients and Methods

This study was conducted at the College of Science, Chemistry Department, Diyala University, and the National Center for the Detection and Treatment of Thalassemia, from December 2024 to June 2025. It involved 81 patients aged between 18 and 38 years with beta-thalassemia major (BTM): 30 with hypogonadism (Group I), 51 without hypogonadism (Group II), The male participants diagnosed with BTM exhibited secondary hypogonadism, as defined by the 2022 criteria from the European Society of Endocrinology (ESE), which require: a total testosterone level of less than 300 ng/dL on two separate occasions, and the presence of at least two symptoms (which may include erectile dysfunction, alopecia, or hirsutism).

2.2 Inclusion Criteria

For the study specified that male patients must be diagnosed with β-thalassemia major, not have undergone splenectomy, and have no history of hormonal drug use. All thalassemic patients were part of a regular blood transfusion program, and the age range for participants was set at 15 to 30 years.

2.3 Exclusion Criteria

Included patients with non-transfusion-dependent β-thalassemia, thalassemia-sickle anemia, and α-thalassemia; female thalassemic patients; and those on hormonal treatment within the previous three months.

2.4 Blood Collection

Ten milliliters of venous blood were collected from each patient and control using plastic disposable syringes after a 12-hour fast. The samples were centrifuged to isolate the serum, which was then divided into sample tubes for the measurement of serum INSL3 levels in the fasting state. Additional biochemical tests were conducted, including assessments of MAD levels, IMA, blood urea, and serum creatinine to evaluate renal function.

2.5 Laboratory Studies

INSL3 levels were measured using a commercial ELISA kit specific for Human INSL3 (ELK Biotechnology, China) ,with intra- and inter-assay coefficients of variation (CVs) below 8%. The concentrations of testosterone, LH, FSH, and inhibin-B were quantified through chemiluminescent immunoassays conducted on automated analyzers (Elabscience Biotechnology Inc., China) [8]. Ferritin, iron, UIBC, and TIBC were assessed using colorimetric methods on an automated biochemical analyzer (e.g., Roche Cobas Integra). Malondialdehyde (MDA) was estimated via the thiobarbituric acid reactive substances (TBARS) assay, which is commonly employed to assess lipid peroxidation and oxidative stress. Ischemia-Modified Albumin (IMA) was measured using the albumin cobalt binding (ACB) test as outlined by Bhagavan, N. V. et al. (2003) [9].

2.6 Statistical Analyses

Statistical analyses were conducted using SPSS version 23 (Statistical Package for Social Sciences). Initially, a frequency distribution for the selected variables was generated. A p-value of less than 0.05 was deemed statistically significant. To assess the statistical significance of the difference in means for a normally distributed quantitative variable between two groups, an independent samples t-test was employed. In cases involving more than two groups, the ANOVA test was utilized. Additionally, multiple regression analysis with 95% confidence intervals was performed to identify predictors of hypogonadism. The significance threshold was established at $p < 0.05$. The area under the ROC curve was used to evaluate the effectiveness of INSL3 in distinguishing between the two patient groups.

3 RESULTS

This study involved 111 subjects, age-matched with a mean age of 22.24 ± 7.78 years. We observed a highly statistically significant difference among all studied groups concerning Ferritin, Iron, UIBC, TIBC, MAD, and IAM. Conversely, serum levels of FSH, LH, Testosterone, INSL3, and Inhibin B were significantly lower in BTM patients with hypogonadism compared to the GII and control groups (see Table 1). The correlation between INSL3 and other parameters in BTM patients is presented in Table 2, revealing a positive correlation between Inhibin B and INSL3 ($r = 0.745$, $p = 0.001$).

Univariate Logistic Regression Analysis (Table 3) identified INSL3, LH, and FSH as independent predictors of hypogonadism. After adjusting for covariates as shown in Table 4, INSL3 was confirmed as an independent predictor with a protective effect, while elevated FSH and LH maintained their status as risk factors. These findings underscore the significance of INSL3 and gonadal hormones in the progression of hypogonadism. Notably, INSL3 exhibited a remarkable ability to detect hypogonadism when compared to testosterone, demonstrating high sensitivity and specificity (≥92.1%) and high accuracy (≥93.2%), as detailed in Table 5 and illustrated in Figures 1a and 1b.

4 DISCUSSIONS

The current study revealed that INSL3 levels were significantly lower in patients with beta-thalassemia major (BTM) compared to the control group. Additionally, BTM patients with hypogonadism exhibited markedly lower INSL3 levels than those without hypogonadism. This finding aligns with previously reported results: Lăptoiu et al. demonstrated that adult men with testicular damage affecting Leydig cells and hypospermatogenesis have reduced INSL3 levels compared to their counterparts with normally descended testes [10]. Similarly, Van Brakel et al. observed low INSL3 levels, although no significant differences were found among the various types of undescended testes. Their research indicated that men whose testes descended spontaneously, without the need for medication or surgical intervention, did not experience altered fertility-assessed through testicular volumetry, hormone levels, and semen analysis-compared to those undergoing orchidopexy following a 'watch and wait' approach during childhood [11]. The decreased INSL3 levels in thalassemia patients with hypogonadism suggest early damage to Leydig cells due to iron accumulation, which induces oxidative stress, as evidenced by high values recorded in the study for MDA and IMA indices. We propose a potential mechanism: increased iron overload leads to heightened oxidative stress, which inhibits Leydig cell function, resulting in decreased INSL3 levels and further contributing to the progression of hypogonadism. This study identified a highly significant positive correlation between INSL3 and inhibin-B. Our findings, consistent with previous research, indicate that the decline in both INSL3 and inhibin B occurs concomitantly in primary hypogonadism, with circulating levels of these two peptides showing a significant correlation [12]. This relationship reflects the overall testicular damage characteristic of this condition. A study by Trabado et al. (2014) further demonstrated a strong positive correlation between serum INSL3 and inhibin B in a cohort of hypogonadal men [13]. This association remained robust even when focusing specifically on men with primary testicular failure, suggesting that the simultaneous declines resulting from testicular failure are mainly independent of testosterone levels [14]. Additionally, significantly reduced INSL3 concentrations have been reported in both adolescents and adult patients with Klinefelter syndrome, a common cause of primary hypogonadism, which is also associated with very low levels of inhibin B [12]. The concurrent downregulation of these biomarkers, which have distinct cellular origins but occur in similar testicular pathological conditions, likely contributes to their strong association.

Table 1: Comparison of parameters among the three studied groups.

Parameters	GI (n=30) mean± SE	GII (n=51) mean± SE	Control groups(n=30) mean± SE	F	Sig
Age	22.8±1.3	21.62±2	23.60 ± 5.22	1.5	0.26
BMI	19.40±	19.8±6028	24.96 ± 2.54	2.74	0.43
Hb (g\dL)	7.024±1.38[a,c]	7.131±3.73[b]	15.29±1.67	11.67	0.05
HCT(PCV)%	21.05±5.17[a]	21.31±7.65[b]	45.49±3.33	19.301	0.034
PLT	473.13±7.9[a]	507.3±92.2[b]	265.83±3.3	11.63	0.043
Ferritin ng\ml	180±2.45[a,c]	167±9.9[a]	127.86 ± 8.27	45.042	0.0001
Iron ug\dl	249.2±6.33[a,c]	219.8±0.8[b]	117.63 ± 5.17	53.192	0.001
UIBC ug\dl	271.06±3.3[a,c]	178.10 ± 6.00[b]	95.05±0.23	2.136	.123
Glucose mg\dl	93.24±6.67	93.48±1.4	88.46 ± 8.56	1.478	.233
Urea (mg\dl)	27.82±1.4	28.8934±0.6	22.67 ± 4.68	8.501	0.234
Creatinine mg\dl	0.433± 0.3	0.511±0.5	0.78± 0.16	53.036	0.53
Testosterone (ng/ml)	2.19±0.67[a,c]	5.431±1.4	5.77 ± 0.97	122.675	0.01
INSL3 (pg\mL)	89.81±2.75[a,c]	163.31±0.21[b]	398.6 ± 31.4	364.015	0.001
MDA(ng\mL)	371.9±1.64[a,c]	402.64±7.6[b]	198.8 ± 30.9	51.540	0.000
INH-b (pg\mL)	43.850±3.3[a,c]	81.55±3.37[b]	153.84 ± 17.5	254.122	9.001
IMA (ng\mL)	5341.47±53[a]	5279.45±43[b]	2191.6± 254.3	37.446	0.0001
LH (mIU\mL)	1.449±0.27[a,c]	5.488±0.43	5.68 ± 0.49	248.373	0.01
FSH (mIU\mL)	1.9 15±0.38[a,c]	4.646±3.1	6.59 ± 0.79	172.824	0.01

a. Referred to signifies differences between G1 and control group;
b. Referred to signifies differences between G2 and control group;
c. Referred to signifies differences between G1 and G1.

Table 2: Correlation between INSL3 and other parameters in all BTM patients.

Characteristic	INSL3	
	r	*P*
Age	-0.45	0.043*
BMI	0.034	0.17
TIBC	-0.084	0.485
UIBC	-0.304	0.006*
Ferritin	0.047	0.646
Iron	-0.703	0.001*
Hb	0.009	0.960
HCT	0.067	0.393
Platelet	0.023	0.840
Glucose	0.038	0.791
Urea	0.046	0.685
Creatinine	0.192	0.089
FSH	-0.053	0.638
LH	-0.018	0.871
Testosterone	-0.002	0.984
IMA	0.053	0.638
INH-b	0.745	0.001*
MDA	0.021	0.850

Table 3: Univariate Logistic regression analysis of indepented pactior of hypogonadism.

Parameters	Area	Std. Error[a]	Sensitivity	specificity	Youden index	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
							Lower Bound	Upper Bound
INSL3	0.893	0.035	92.1	93.2	0.853	0.000	0.823	0.962
Testosterone	0.790	0.056	88.3	91.6	0.749	0.000	0.684	0.903
The test result variable(s): INSL has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.								
a. Under the nonparametric assumption, b. Null hypothesis: true area = 0.5								

Table 4: Multivariate logistic regression analysis of independent predictors of hypogonadism.

Parameters	Unstandardized Coefficients		Standardized Coefficients	t	p-value
	B	Std. Error	Beta		
Age	.388	.579	.050	.671	.505
Ferritin ng\ml	.001	.001	.033	.474	.637
Iron ug\dl	-.006-	.028	-.014-	-.199-	.843
UIBC ug\dl	-.002-	.004	-.044-	-.633-	.529
TIBC ug\dl	.007	.018	.028	.373	.710
Glucose mg\dl	.020	.042	.036	.487	.628
Urea (mg\dl)	-.143-	.284	-.040-	-.506-	.615
Creatinine mg\dl	5.422	11.516	.036	.471	.639
Testosterone (ng/ml)	4.559	5.491	.115	.830	.409
INSL3(pg/dl)	.362	.032	.817	11.220	.000
MDA(ng\mL)	.006	.025	.026	.243	.809
INH-b (pg\mL)	.001	.002	.060	.447	.657
IMA (ng\mL)	-6.062-	11.699	-.082-	-.518-	.606
LH (mIU\mL)	1.757	.852	.710	2.062	.043
FSH (mIU\mL)	4.559	5.491	.830.	115	.009

Table 5: AUC and validity of INSL3 and Testosterone to differentiate between patients themselves.

Variable	Adjusted OR	95% CI	P-value
INSL3	0.876	0.812-0.954	<0.001
LH	1.451	1.182-1.723	<0.001
FSH	1.001	1.000-1.002	0.037

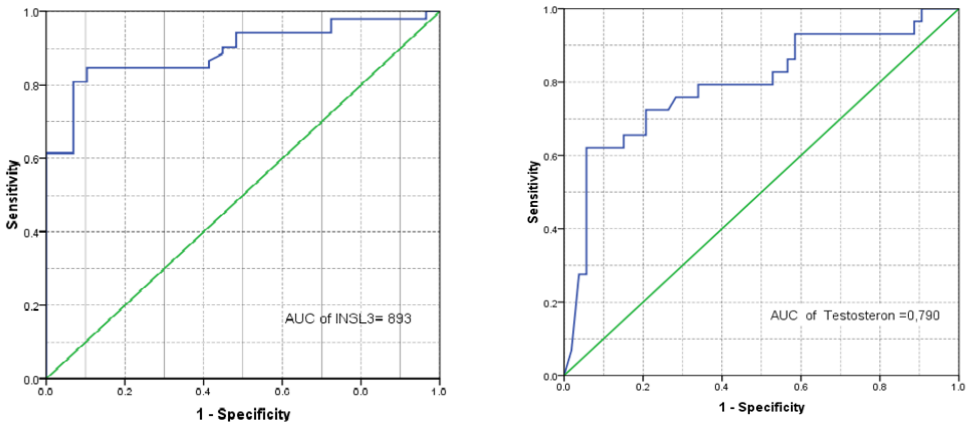

Figure 1: Receiver operating characteristic (ROC) curve analysis of INSL3 and testosterone for differentiation of hypogonadism in BTM patients: a) ROC curve for INSL3; b) ROC curve for testosterone.

In our study, both univariate and multivariate logistic regression analyses identified INSL3, LH, and FSH as independent factors influencing hypogonadism. This condition is the most commonly reported endocrine complication among patients with thalassemia major, affecting 70–80% of this population. Hypogonadism is likely attributed to iron deposits in the gonads, pituitary gland, or both. However, hypogonadotropic hypogonadism caused by iron deposition in the pituitary gonadotrope is more frequently observed. Gonadal iron deposition in the ovaries or testes occurs less often, leading to significant impairment in the synthesis of luteinizing hormone (LH) and follicle-stimulating hormone

(FSH) [15,16]. Furthermore, INSL3 levels are influenced by the long-term cytotropic effects of the hypothalamic-pituitary-gonadal axis, in contrast to testosterone, which responds acutely to luteinizing hormone (LH) stimulation [17]. INSL3 is directly related to the quantity and differentiation of Leydig cells, making it an ideal marker for assessing Leydig cell function. Notably, INSL3 is more sensitive than testosterone when there is Leydig cell impairment, and a decrease in INSL3 levels in adult men can serve as an early indicator of endocrine testicular dysfunction [18]. Based on our findings, the measurement of INSL3 levels should be considered in the clinical management of male hypogonadism and in evaluating testicular endocrine function. The ROC analysis further supports the diagnostic usefulness of INSL3, demonstrating performance comparable to, or even superior to, traditional markers.

Our report presents several strengths. Firstly, we ensured an age match among the three groups. Secondly, both hypogonadism patients and those without hypogonadism were administered the same medications. Thirdly, the two patient groups received only one type of treatment. Finally, we organized the patients into subgroups based on the presence of hypogonadism. However, this research does have some limitations. The sample size was relatively small, and a detailed follow-up analysis assessing INSL3 levels from the time of diagnosis to the onset of hypogonadism in BTM may provide a deeper understanding of INSL3's function and its potential as a marker or therapeutic target.

5 CONCLUSIONS

This study demonstrates that serum INSL3 levels are significantly reduced in male patients with beta-thalassemia major (BTM) complicated by hypogonadism. Moreover, INSL3 showed a strong positive correlation with inhibin B, supporting its close relationship with testicular Sertoli–Leydig cell function.

Multivariate analysis confirmed that INSL3 acts as an independent predictor of hypogonadism, even after adjustment for iron overload and oxidative stress markers. Importantly, ROC curve analysis revealed excellent diagnostic performance of INSL3 (AUC = 0.893), with high sensitivity and specificity, outperforming traditional hormonal markers such as testosterone.

These findings suggest that INSL3 may represent a promising early biomarker for detecting Leydig cell dysfunction and subclinical hypogonadism in patients with BTM. Given the role of iron overload and oxidative stress in testicular impairment, INSL3 could also reflect early gonadal damage before overt testosterone decline becomes evident.

Overall, INSL3 has potential clinical utility in improving early diagnosis and monitoring of reproductive endocrine dysfunction in BTM patients. However, larger longitudinal studies are required to validate its prognostic value and clarify its mechanistic role in the hypothalamic–pituitary–gonadal axis under conditions of chronic iron overload.

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