Journal of Endocrinological Investigation

Thyroid function in Klinefelter syndrome: a multicenter study from KING group

--Manuscript Draft--

Manuscript Number:	JENI-D-18-00755R1
Full Title:	Thyroid function in Klinefelter syndrome: a multicenter study from KING group
Article Type:	Original Article
Funding Information:	
Abstract:	Purpose The prevalence and the etiopathogenesis of thyroid dysfunctions in Klinefelter syndrome (KS) are still unclear. The primary aim of this study was to evaluate the pathogenetic role of hypogonadism in the thyroid disorders described in KS, with the scope to distinguish between genetic alterations or hormonal involvement. Therefore, we evaluated thyroid function in KS and in non-KS hypogonadal patients. Methods This is a case-control multicentre study from KING group: Endocrinology clinics in university-affiliated medical centres. One hundred seventy four KS, and sixty-two non-KS hypogonadal men were enrolled. The primary outcome was the prevalence of thyroid diseases in KS and in non-KS. Changes in hormonal parameters were evaluated. Exclusion criterion was secondary hypothyroidism. Analyses were performed using Student's t test. Mann-Whitney test and chi-square test. Results FT4 was significantly lower in KS vs non-KS. KS and non-KS presented similar TSH and testosterone levels. Hashimoto's thyroiditis (HT) was diagnosed in 7% of KS. Five KS developed hypothyroidism. The ratio FT3/FT4 was similar in both groups. TSH index was 1.9 in KS and 2.3 in non-KS. Adjustment for differences in age, sample size and concomitant disease in multivariate models did not alter the results. Conclusions We demonstrated in KS no etiopathogenic link to hypogonadism or change in the set point of thyrotrophic control in the altered FT4 production. These results suggest possible deiodinase impairment. The prevalence of HT in KS was similar to normal male population, showing absence of increased risk of HT associated with the XXY karyotype.
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Author Comments:	We complete and sign the Authorship Change Form, Sincerely yours Daniela Pasquali
Response to Reviewers:	Comments and answers Reviewer #1: In this paper titled "Thyroid function in Klinefelter syndrome (KS): a multicenter study from KING group", the authors compared thyroid function in a group of patients with Klinefelter with a group of hypogonadal patients. The aim of the study is to clarify the prevalence and etiology of hypothyroidism in KS patients. Thyroid function was studied using serum hormonal and antibody concentration. The authors conclude that there is no etiopathogenic link between KS and hypothyroidism, and that the KS patients present an impaired production of T4 by the thyroid gland, with an adaptive type II deiodinase activity that allows a normal peripheral circulating amount of FT3 and normal TSH levels. In the abstract, page 2 line 19 (it is in line 9): the number of KS patients (174) does not coincide with the number in table 1 (176). The correct number of KS patients is 174. This has been corrected here and throughout the manuscript. Page 4, lane 2: Authors should check that the number of KS patients declared (174) is correct. The correct number of KS patients is 174. This has been corrected here and throughout the manuscript. The same check should be made at every point where this number is being reported [page 4 line 19 (this in in line 9) and table 1]. The correct number of KS patients is 174. This has been corrected here and throughout the manuscript. Page 7 line 17 The authors should consider the following review: Mol Cell Endocrinol. 2018 May 15;467:49-59. doi: 10.1016/j.mce.2017.11.006 as a introduction to the behavioral impact, if any, of the slight TH derangements here found (see also: Endocrine. 2015 Jun;49(2):333-8. doi: 10.1007/s12020-014-0520-7. and Mol Hum Reprod. 2010 Jun;16(6):418-24. doi: 10.1093/molehr/gaq022.) highlighting that the ejaculatory control has never been studied in KS. We considered these article and, as requested, included them in the discussion. Authors may consider to delete the figure 1 that is not significant and to replace it with a figure of FT4 levels, which are significantly different between the two groups analyzed. We modified the figures (Figure 1, 2 and 3), as requested. Reviewer #2: The manuscript by Dr Balercia et al entitled "Thyroid function in Klinefelter syndrome: a multicenter study from KING group" investigates the thyroid function in a relevant number of KS patients. This research finds that the prevalence of thyroid autoimmune disorders in KS patients is not different from the general population and that KS patients show mildly reduced FT4 levels compared to another hypogonadic control group. This is a multicentric study, conducted by specialists who

	are experts of KS. These findings are somehow confirmatory of previous studies. Major criticisms 1) It is unclear how the work is designed to distinguish between "genetic alterations or hormonal involvement". This sentence should be rephrased. The sentence was reworded to enhance its clarity. 2) Taking into account that FT4 measurement is so critical for the results of this work, the percentage of the different assays and manufacturer employed to measure FT4 should be indicated in the methods section. We reported the coefficient of variation for FT4 assay in Methods (Daniela per favore vedi il testo). 3) If “an altered T4 production by the thyroid gland” is present and TSH levels are not increased (compared to controls) this suggests a different set point of the thyroid axis in the two experimental groups. This hypothesis is more plausible than alterations based only on increased D2 activity. We agree with the Reviewer. Therefore, and we removed all the discussion on deiodinase from the Abstract and the Discussion, considering it as a possible starting point for further studies. 4) To interpret adequately thyroid function data, iodine intake and urinary iodine should have been measured. This is an important limitation of the study. We included these comments among possible future research development in KS patients. 5) From the results section it is unclear if the prevalence of TH, in the two experimental groups, is different or not. Since this is not the case, the data can be reported in table 1. We reported the prevalence of TH in Table 1. 6) The involvement of D2 activation in this context is not enough supported by the results or by previous findings. For this reason, this speculation, should be removed from the abstract and discussion. We agree with the Reviewer. Therefore, and we removed all the discussion on deiodinase from the Abstract and the Discussion, considering it as a possible starting point for further studies. See also Q3.
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Thyroid function in Klinefelter syndrome: a multicenter study from KING group

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***Disclosure summary*:** The authors have nothing to disclose

Acknowledgments This study was performed on behalf of the Klinefelter ItaliaN Group (KING). Coordinators: Antonio Aversa (Catanzaro), Giancarlo Balercia (Ancona), Marco Bonomi (Milan), Aldo Calogero (Catania), Giovanni Corona (Bologna), Francesco Giorgino (Bari), Andrea Fabbri (Rome), Alberto Ferlin (Padua), Emanuele Ferrante (Milan), Felice Francavilla (L'Aquila), Vito Giagulli (Conversano, Bari), Emmanuele Jannini (Rome), Fabio Lanfranco (Turin), Mario Maggi (Florence), Daniela Pasquali (Naples), Rosario Pivonello (Naples), Alessandro Pizzocaro (Milan), Antonio Radicioni (Rome), Vincenzo Rochira (Modena), Linda Vignozzi (Florence). Members: Marco Barchi (Rome), Biagio Cangiano (Milan), Rosita A. Condorelli (Catania), Giuliana Cordeschi (L'Aquila), Settimio D'Andrea (L'Aquila), Antonella Di Mambro (Padua), Carlo Foresta (Padua), Sandro Francavilla (L'Aquila), Andrea Garolla (Padua), Lara Giovannini (Ancona), Antonio R.M. Granata (Modena), Sandro La Vignera (Catania), Giovanna Motta (Turin), Luciano Negri (Milan), Fiore Pelliccione (Milan), Luca Persani (Milan), Ciro Salzano (Naples), Daniele Santi (Modena), Riccardo Selice (Padua), Manuela Simoni (Modena), Carla Tatone (L'Aquila), Giacomo Tirabassi (Ancona), Alberto Stefano Tresoldi (Milan) and Enzo Vicari (Catania). The KING belongs to the Italian Society of Andrology and Sexual Medicine (SIAMS) and aims to promote all the activities, clinical, research, and informative, concerning KS in Italy.

Keywords: Klinefelter syndrome, Testosterone, Hypergonadotropic hypogonadism, Thyroid hormones, Thyroid diseases, Hashimoto's thyroiditis

Abstract

Purpose: The prevalence and the etiopathogenesis of thyroid dysfunctions in Klinefelter syndrome (KS) are still unclear. The primary aim of this study was to evaluate the pathogenetic role of hypogonadism in the thyroid disorders described in KS, with the scope to distinguish between patients with KS and hypogonadism due to other causes (Kallmann syndrome, idiopathic hypogonadotropic hypogonadism, iatrogenic hypogonadism and acquired hypogonadotropic hypogonadism after surgical removal of pituitary adenomas) called non-KS. Therefore, we evaluated thyroid function in KS and in non-KS hypogonadal patients.

Methods: This is a case-control multicentre study from KING group: Endocrinology clinics in university-affiliated medical centres. One hundred seventy four KS, and sixty-two non-KS hypogonadal men were enrolled. The primary outcome was the prevalence of thyroid diseases in KS and in non-KS. Changes in hormonal parameters were evaluated. Exclusion criterion was secondary hypothyroidism. Analyses were performed using Student's t test. Mann-Whitney test and chi-square test.

Results: FT_4 was significantly lower in KS vs non-KS. KS and non-KS presented similar TSH and testosterone levels. Hashimoto's thyroiditis (HT) was diagnosed in 7% of KS. Five KS developed hypothyroidism. The ratio FT3/FT4 was similar in both groups. TSH index was 1.9 in KS and 2.3 in non-KS. Adjustment for differences in age, sample size and concomitant disease in multivariate models did not alter the results.

Conclusions: We demonstrated in KS no etiopathogenic link to hypogonadism or change in the set point of thyrotrophic control in the altered FT4 production. The prevalence of HT in KS was similar to normal male population, showing absence of increased risk of HT associated with the XXY karyotype.

Introduction

Klinefelter Syndrome (KS) is the most frequent observed sex chromosomal anomaly, with an estimated frequency of 1:500 to 1:1000 men (1). The classic form of KS, which is present in the 80–90 % of the cases, is defined by a 47,XXY karyotype, low serum testosterone (T) coupled with elevated gonadotropins, small and firm testes, azoospermia, and tall stature. The clinical presentation of KS syndrome is widely heterogeneous. Only about one in 10 males with KS is diagnosed before puberty and 25-50% in adult age, often during infertility investigation. KS is associated with several clinical conditions coming from both the genetic abnormalities, and hypogonadism. KS patients present several comorbidities including cardiovascular diseases, osteoporosis, diabetes mellitus, and metabolic syndrome, leg ulcers, mostly related to hypogonadism (2-5). Consequently, KS males need lifelong testosterone replacement treatment. Yet, the therapy does not counteract or prevent all comorbidities. It is still unclear whether the extra chromosome X adds an extra testosterone-independent feature to the complexity of KS comorbidities. Thus, the relationship between clinical phenotype, endocrine dysfunction, KS comorbidities and genetic background needs further investigations (1). Various thyroid abnormalities were described, mostly in old studies based on few participants, the most common being a low response in serum TSH to a TRH stimulation test (6,7). KS had a general shift toward lower values in distribution of serum FT4 with no compensatory increase in serum TSH (8). The most likely mechanism suggested is a decrease or change in set point of thyrotrophic control of thyroid function (8). Recently a study demonstrated that pubertal KS patients have significantly lower FT3 serum levels than healthy age-matched boys, whereas TSH and FT4 are in the normal reference range (9). Furthermore, some authors have recently analysed KS comorbidities using data from the Danish National Patient Registry (10). They searched for differentially expressed genes in peripheral blood and found several expression signatures that correlated with KS status either

positively or negatively. Genes involved in 'abnormal thyroid hormone metabolism' were upregulated (10).

To better clarify the prevalence of thyroid diseases and the role of 47XXY as well as the hypogonadism in thyroid dysregulation, we studied thyroid function in one hundred seventy four patients with KS compared with non-KS hypogonadal men.

Subjects and Methods

Patient population

We performed a multicentre case control-study, involving the national network of academic or general hospitals named KING (Klinefelter ItaliaN Group). The entire cohort consisted of one hundred seventy four consecutive KS subjects, and sixty-two age-matched non-KS hypogonadal men recruited among those regularly attending KING centres since 2015. Inclusion criteria were: i) a verified KS karyotype (47, XXY); ii) a written informed consent. Patients underwent a complete medical and family history evaluation. We examined thyroid function, and thyroid antibodies (Ab). Subjects, who had elevated plasma TPOAb and TgAb above 350 IU/ml, and thyroid parenchyma heterogeneity with reduced echogenicity, were considered as Hashimoto's thyroiditis (HT) patients. Five KS patients were already under l-thyroxine replacement therapy.

Collection of samples

Venous blood (2 mL) from participants was collected by blood banks of the hospitals of the KING centres during the same period of KS and non-KS patients' recruitment. Samples, blind of identification, treatment or placebo group, were immediately sent for analysis of TSH, FT4, FT3, TPOAb and TgAb and total testosterone to the laboratory facility in each hospital institution of the KING groups. The measurements were done on the same day in primary tubes, after blood centrifugation at 3200 RPM for 15 min.

Biochemical data

As we recruited patients regularly attending KING centres, different methods have been used. In the majority of the cases, serum TSH, FT4, FT3 concentrations were measured by chemiluminescent immunometric assay (Roche Diagnostics GmbH, Mannheim, Germany). The coefficient of variation was 5.4% for the FT4 assay. The manufacturer's reference limits are TSH (0.35–5.5 mIU/L), FT4 (10.2–31 pmol/L), and FT3 (3.5–6.5 pmol/L). Detection limit for TSH is 0.005 μIU/mL, functional sensitivity 0.014 μIU/mL. Thyroid function alteration was classified according to the American Thyroid Association guidelines (11, 12). Clinically overt hyperthyroidism, undetectable to less than 0.1mIU/L TSH, more than normal range FT3, and FT4; subclinical hyperthyroidism, undetectable to less than 0.1 mIU/L TSH and FT3 and FT4 in the normal range without exogenous T4 intake; clinically overt hypothyroidism, TSH above the upper limit of the normal range (5mIU/L in our assay) and FT4 below 10.2 pmol/L; and subclinical hypothyroidism, TSH above the upper limit of the normal range and FT4 in the normal range (11,12). Total testosterone was measured by immunoassay using a commercial Kit, automated immunoassays (DiaSorin Liaison, Saluggia, VC, Italy).

Exclusion criteria

KS patients with mosaic forms of chromosomal aneuploidy or any other structural or numerical karyotype anomaly, with AZF microdeletions, were excluded. Moreover, exclusion criterion in non-KS control group was also secondary hypothyroidism defined as undetectable to less than 0.1 mIU/L TSH, FT4, and FT3 in the lower range without exogenous T4 intake. Subjects with clinically overt thyroid dysfunction, or personal or family history of thyroid disease were excluded from the control sample, which was, therefore, a population of clinically normothyroidal individuals.

Statistical analysis

Data were tabulated on a spreadsheet and analysed with SPSS statistics 17 software. We calculated mean and standard deviation for continuous variables.

Analyses were performed using Student's t test. TSH and testosterone were analysed using Mann-Whitney test and, for TPO antibodies chi-square test analysis was performed.

All analysis was two-sided and p values minor 0.05 were considered statistically significant.

This study was approved by "Comitato Etico Seconda Università degli studi di Napoli- Azienda Ospedaliera Universitaria SUN-AORN Ospedale dei Colli" n 1489, 26.10.2015 to DP.

Results

Demographic characteristics of the study groups

The mean age at study entry was 40.6 ± 1 (mean ± SD; range, 16–59) and 40.0 ± 2.4 (mean ± SD; range, 16–71) years in KS and in non-KS respectively. The non-KS group was composed by thirty-three Kallmann syndrome patients, seventeen patients with idiopathic hypogonadotropic hypogonadism, four patients were affected by iatrogenic hypogonadism, and eight by acquired hypogonadotropic hypogonadism after surgical removal of pituitary adenomas (table 2). The comparison between KS and non-KS group allowed evaluating the impact of the hypogonadal condition and the genetic origin of thyroid function alterations in KS. All the subjects were under testosterone replacement treatment. T levels were similar in both KS (324±69 ng/dl) and non-KS (284.7±36 ng/dl), and in both groups at the lower end of the normal range.

Hashimoto's thyroiditis in KS vs. non-KS patients

In KS group 7% of the patients showed TPOAb positivity, while 4% out of sixty-two hypogonadal non-KS group had HT (Table 1). Five KS with HT were diagnosed with hypothyroidism and treated with L-Thyroxine substitutive therapy.

Hypothalamic–pituitary–thyroid axis dysregulation in KS

Serum FT4 was significantly lower in men with KS versus non-KS [mean (SD): 10.69 (0.2) *vs.* 11.7 (0.4) pg/ml; $p < 0.006$] (Table 1). The FT3/FT4 ratio was similar in KS and in non-KS group (0.32 vs. 0.29, p<0.06) (Figure 2). Nevertheless, KS and non-KS patients showed similar TSH and total T

levels [TSH: mean (SD): 1.61 (0.87) *vs.* 1.75 (1.2) p= 0.1; testosterone mean (SD): 324 (69) *vs.* 284 (36) ng/dl respectively p = 0.2] (Table 1). We calculated TSH Index, an accurate estimation of the pituitary dysfunction severity in patients with hypopituitarism (14), that was not statistically different between KS compared to non-KS (1.9 vs. 2.1) (Figure 3). Adjustment for differences in age, sample size and concomitant disease in multivariate models did no alter the results.

Discussion

The etiopathogenesis of thyroid dysfunction in KS is still under debate. Here we show lower FT4 in KS, with no changes in FT3 and TSH levels. We have not found a higher prevalence of HT in KS. The cross talk between thyroid and gonadal hormones is well known. Thyroid hormone regulation of sex steroid synthesis and action in both the brain and gonads are important for gonad development and brain sexual differentiation (15, 16). Thyroid transcription factor-1, a homeobox gene that regulates the transcriptional activity of thyroid-specific genes in thyroid follicular cells, occupies a central position within gene networks involved in the hypothalamic control of GnRH production (17). The effects of thyroid hormones in men and their correlations with sexual behaviour are not yet clear even though several studies show an association between thyroid diseases and altered sexual function (hypoactive sexual desire disorder, premature and delayed ejaculation, and erectile dysfunction) (18). KS patients have sexual dysfunctions not specifically associated with the syndrome but related to hypogonadism (19) although the ejaculatory control in this category of patients has not yet been studied.

To elucidate if the thyroid dysfunction in KS is related to the peculiarity of 47XXY condition and/or to T deficiency status, we analysed the occurrence of thyroid diseases in KS and in non-KS hypogonadal men. We have utilized data from a national multicentre study, to overcome the main problem arising from KS studies, the small sample size, that makes difficult to draw strong clinical conclusions. We confirm that males with KS have a shift toward lower values in distribution of serum FT4 (8). In particular, we have found a significant reduction of FT4 level in KS compared to

non-KS, with no statistical differences in TSH and FT3 levels. To verify the hypothesis of an altered set point of thyrotrophic control of thyroid function in KS (8), we have performed TSH index calculation. TSH index is an "FT4-adjusted TSH" that corrects for any physiological TSH suppression, to provide a true estimate of pituitary thyrotrophic function and any pathological pituitary suppression (14). TSH index was not statistically different in KS and non-KS, suggesting an adequate hypothalamic-pituitary-thyroid axis control. We could speculate that, since in healthy and iodine-sufficient individuals, the majority of thyroid hormone produced is T4, synthesized exclusively by the thyroid gland, with a smaller amount (~20%) of T3, which is produced by the thyroid and in peripheral tissues via deiodination of circulating T4, KS patients could have an altered T4 production by the thyroid gland. The novel approach with the comparison of FT4 levels between KS and non-KS patients give us the opportunity to clarify that T deficit, independently of when it started, cannot be considered as a causal determinant in the pathophysiology of this condition. Metabolic syndrome and insulin resistance are specific features of KS (1-3, 20-22) and FT3/FT4 ratio has a better predictive power for metabolic syndrome than TSH (23). We found comparable FT3/FT4 ratio both in KS and in non-KS, suggesting a similar predisposition to increased risk of metabolic syndrome parameters and insulin resistance. Then we took the opportunity to study the prevalence of Hashimoto's thyroiditis (HT) among KS that resulted to be 7.0%. The prevalence of HT in KS was similar to that reported in healthy male volunteers in the Pescopagano survey (24,25). In fact, in this comprehensive study of thyroid disease in Italy 17.3 % of women and 7.0 % of male were affected by HT (24,25). The X chromosome contains the largest number of immune-related genes of the whole human genome (26). Skewed X chromosome inactivation has been considered one of the key factors that influence the female predisposition to autoimmunity (27). Laboratory studies have shown high levels of autoantibodies to the thyroid gland among people with KS (28). An English national cohort study of people with KS, found significantly increased risks of several autoimmune diseases, including acquired hypothyroidism (29). The extra X chromosome could predispose KS to higher prevalence of HT. Our Italian

national cohort results do not support this hypothesis and are in contrast with previous studies, in particular considering the prevalence of HT in our KS cohort. This could be explained by the limits of a dataset from hospital discharge records and/or the diagnostic criteria of HT (28,29). Furthermore, a recent study based on data from the Danish National Patient Registry (10), analysed KS comorbidities, searching for differentially expressed genes in peripheral blood. There were genes causative of several Online Mendelian Inheritance in Man (OMIM) diseases such as 'abnormal thyroid hormone metabolism', 'epilepsy' and 'deafness'. These data indicate that more factors are involved in thyroid dysregulation in KS other than increased risk of autoimmune diseases. In previous studies, the most likely mechanism of thyroid abnormalities found in KS is an alteration in set point of thyrotrophic regulation of thyroid function (6-9). It is quite difficult to establish what are the causes and the effects of thyroid dysfunction in KS. However, we can exclude the influence of hypogonadism leading to a dysregulation of hypothalamic–pituitary–thyroid axis.

In conclusion, our study indicates that KS present an impaired production of T4 by the thyroid gland, suggesting a different set point of the thyroid axis versus patients with hypogonadism due to other causes. No evidences of etiopathogenetic link to the hypogonadal status or change in set point of thyrotrophic control were found. Further studies are needed to better understand the underlying mechanism(s) of thyroid dysregulation that may include evaluation of deiodinase activity, iodine intake and urinary iodine excretion.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

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Legends to figures

Figure 1. FT4 levels in Klinefelter syndrome vs. non-Klinefelter syndrome

Figure 2. FT3/FT4 ratio in Klinefelter syndrome vs. non-Klinefelter syndrome. Data are expressed as mean ± SD

Figure 3. TSH index in Klinefelter syndrome vs. non-Klinefelter syndrome. Data are expressed as Mean ± SD.

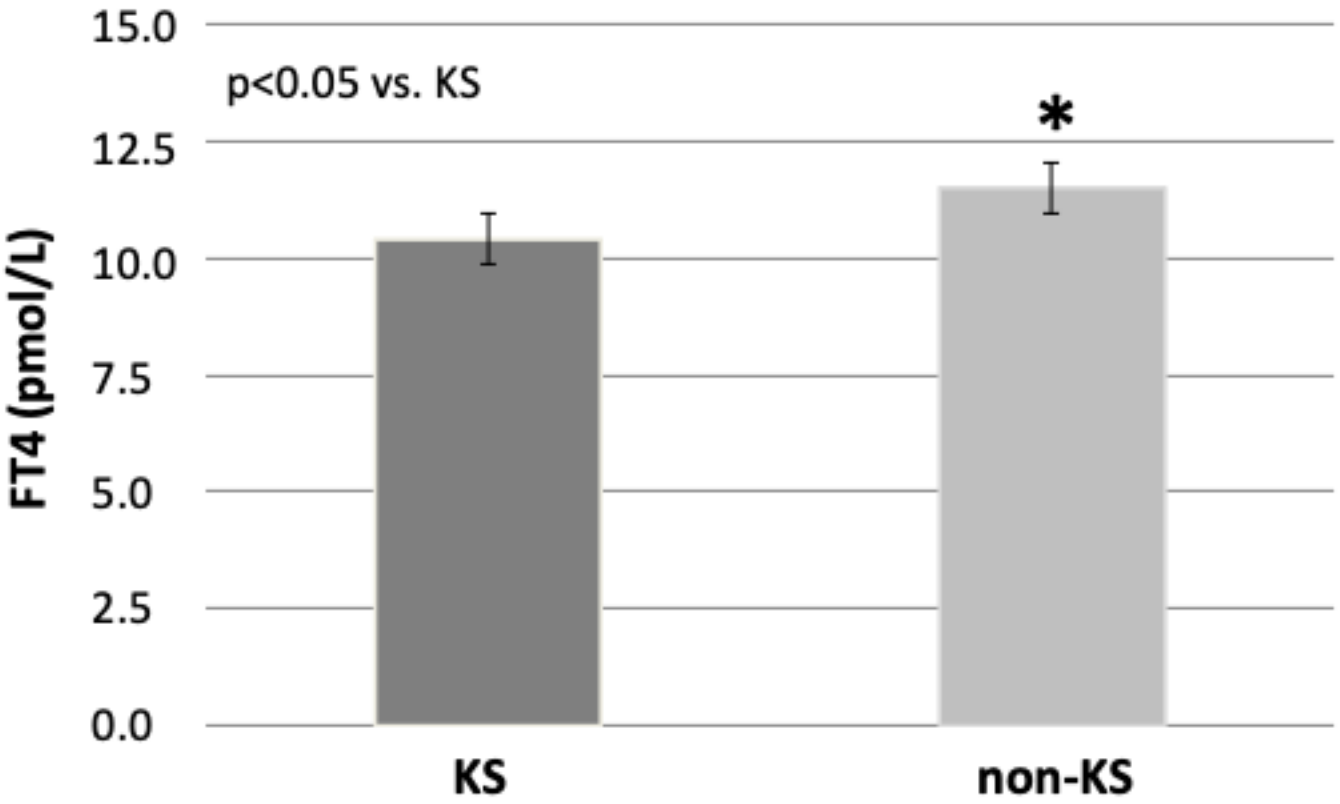

Figure 1

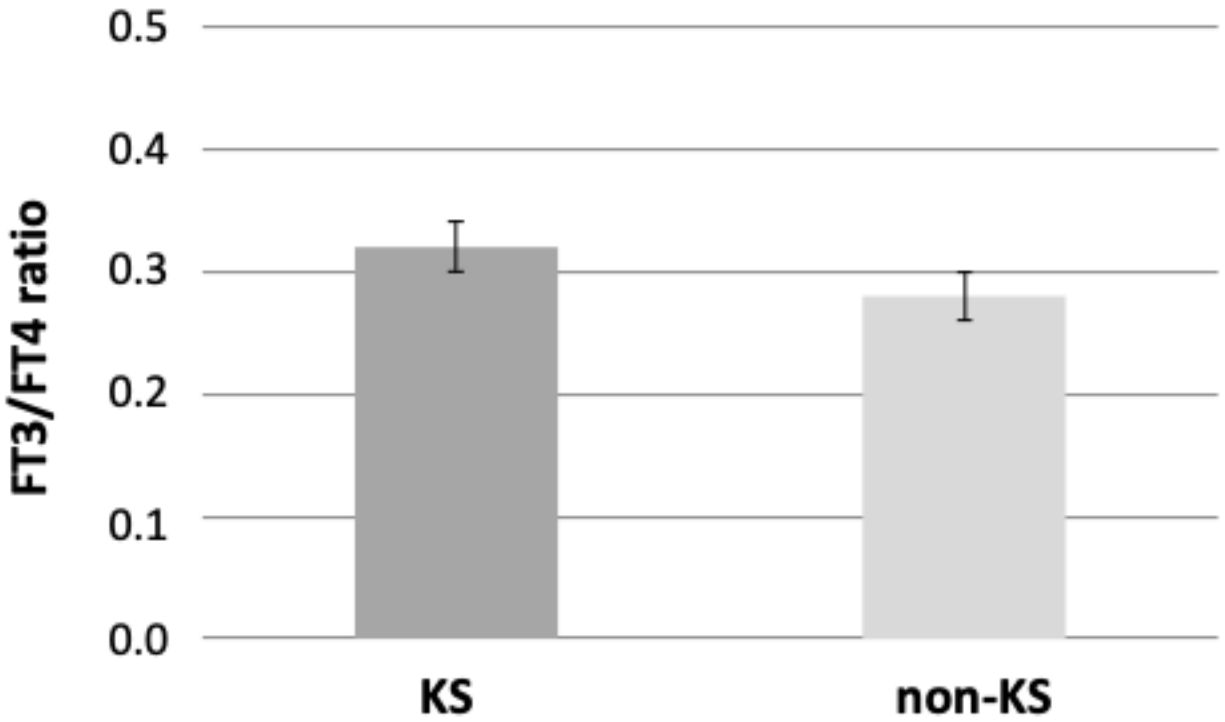

Figure 2

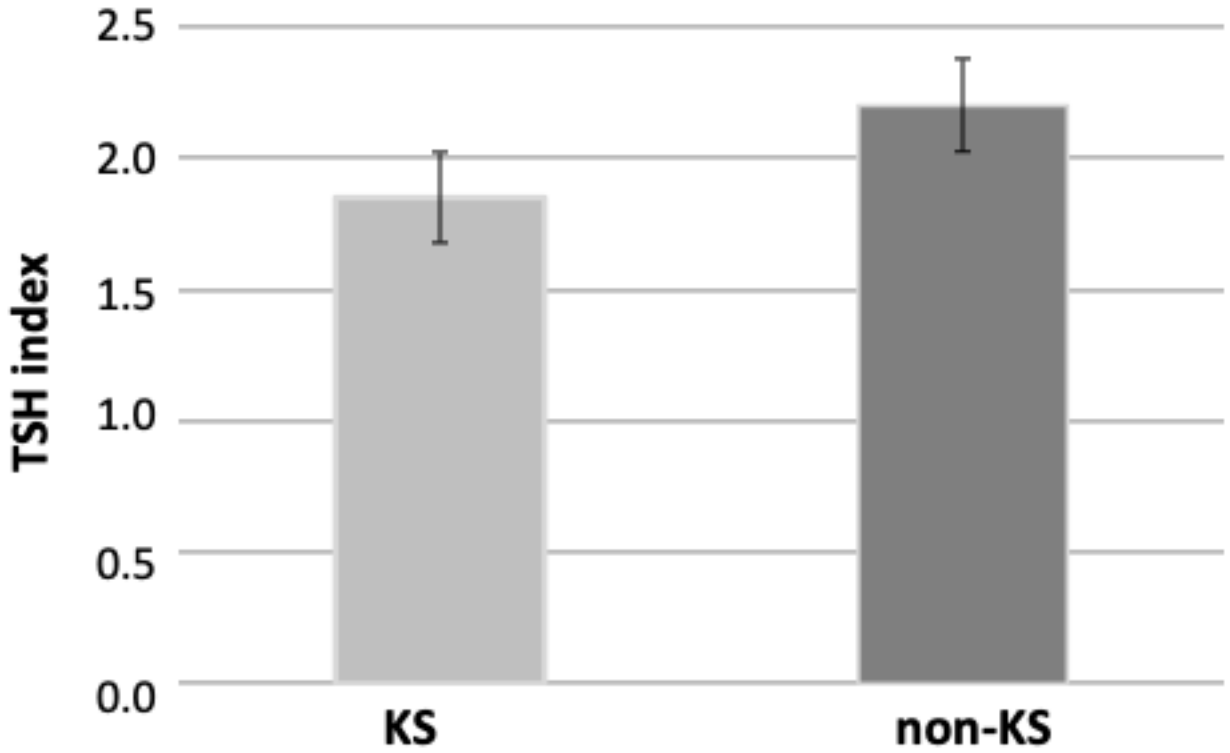

Figure 3

Table 1. Clinical, demographical and hormonal data of subjects with Klinefelter syndrome (KS) or with hypogonadism due to other causes (non-KS).

	KS	non-KS	p
Number of patients	174	62	
Hashimoto's thyroiditis (prevalence)	7%	4%	
Age (years)	40.6 ± 1.1	40.0 ± 2.4	ns
Testosterone (ng/dl)	324.3 ± 69	284.7 ± 36.5	0.05
TSH (IU/L)	1.83 ± 0.1	2.02 ± 0.1	ns
FT3 (pmol/L)	3.3± 0.8	3.2 ±0.1	ns
FT4 (pmol/L)	10.6 ± 0.3	11.7 ± 0.4	< 0.006

Data are reported as mean ± SD; ns= Not significant

Table 2. Diagnoses of patients with hypogonadism not due to Klinefelter syndrome (control group)

	Number of cases
Kallmann syndrome	33
After pituitary surgery	8
Idiopathic	17
Iatrogenic	4
Total	62

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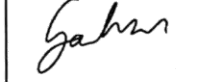
1st author	Giancarlo	Balercia		Division of Endocrinology, Department of Clinical and Molecular Sciences, Umberto I Hospital, Polytechnic University of Marche, Ancona, Italy	14.3.2019
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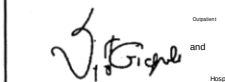
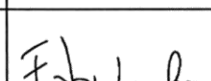
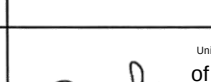
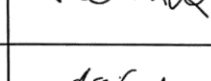
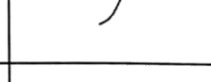
3rd author	Vito A	Giagulli		Outpatient Clinic for Endocrinology and Metabolic Diseases, Conversano Hospital, Conversano, Bari, Italy	14.3.2019
4th authors	Fabio	Lanfranco		Division of Endocrinology, Diabetology, and Metabolism, Department of Medical Sciences, University of Turin, Turin, Italy	14.3.2019
5th author	Vincenzo	Rochira		Unit of Endocrinology, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy	14.3.2019
6th author	Antonio	Giambersio		Healt Center "Maria Teresa di Calcutta" Potenza, Italy	14.3.2019
7th author	Giacomo	Accardo		Department of Advanced Medical And Surgical Sciences, University of Campania "Luigi Vanvitelli", Naples, Italy	14.3.2019

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