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Clinical Consequences of Variable Results in the Measurement of Free Thyroid Hormones: Unusual Presentation of a Family with a Novel Variant in the *THRB* Gene Causing Resistance to Thyroid Hormone Syndrome

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Established Facts

- Resistance to thyroid hormone (RTHβ) syndrome is caused by dominant negative variants in the *THRB* gene.
- Pathogenic variants recur within 3 hot spots in the ligand-binding domain involving the amino acid residues 234–264, 316–347, and 426–454 of thyroid hormone receptor beta.
- Spurious hyperthyroxinemia due to thyroid function assay interferences is a frequent pitfall in the differential diagnosis of central hyperthyroidism.

Novel Insights

- Strict phenotype-genotype correlation and functional studies support p.L428V as a novel dominant negative *THRB* variant, thus expanding the spectrum of gene variants causing RTHβ.
- The in vivo, rather than in vitro, bioassays may be required to reveal the dominant negative action of certain *THRB* variants.
- This report highlights that some assay platforms may underestimate TH levels and may delay the correct diagnosis of RTHβ.
- The degree of sensitivity to thyroid hormone feedback can be highly variable even in RTHβ patients belonging to the same family, with some cases that can be disclosed by the failure to suppress TSH during levothyroxine treatment for nodular goiter.

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Abstract
Introduction: Resistance to thyroid hormone β (RTHβ) is an inherited syndrome caused by dominant negative variants in the *THRB* gene (NM_000461.5). The clinical picture of RTHβ is variable, and patients harboring the same variant may display different degrees of disease severity. ***Case Presentation:*** A 30-year-old man presented with thyrotoxicosis and central hyperthyroidism and was found to have a novel variant in the exon 10 of *THRB* gene (c.C1282G, p.L428V), located within the third hot spot region of the C-terminal of the receptor. Surprisingly, the same variant was found in two other relatives with an apparent normal thyroid function at initial screening. After exclusion of a TSH-secreting adenoma and serum interference in the proband, and the finding that exogenous levothyroxine failed to suppress the TSH in the brother affected by nodular goiter, relatives' thyroid function tests (TFTs) were reassessed with additional analytical method revealing biochemical features consistent with RTHβ in all carriers of the p.L428V variant. Functional studies showed a slightly impaired in vitro transcriptional activity of p.L428V. Interestingly, the expression of the human p.L428V thyroid hormone receptor beta in the zebrafish embryo background generated a phenotype consistent with RTHβ. ***Conclusion:*** Variable results of TFTs on some immunoassays can be a cause of RTHβ diagnostic delay, but the genotype-phenotype correlation in this family and functional studies support p.L428V as a novel *THRB* variant expanding the spectrum of gene variants causing RTHβ. In vivo, rather than in vitro, functional assays may be required to demonstrate the dominant negative action of *THRB* variants.

Introduction

Thyroid hormone resistance syndrome beta (RTHβ) is a rare autosomal disorder caused mainly by dominant negative heterozygous variants in the *THRB* gene (NM_000461.5), encoding for the thyroid hormone receptor β (TRβ) [1, 2]. The most frequent genetic variants associated with RTHβ are single nucleotide changes or small in/dels involving 3 CpG-rich regions located in the ligand binding domain and in the contiguous hinge region [3].

Mutant TRβs retain the ability to bind the DNA and dimerize with retinoid X receptor (RXR) but display either a reduced affinity for T3 or an impaired interaction with the cofactors (coactivators and corepressors), thus losing their ability to modulate target gene expression in different tissues [4]. Inappropriate findings in the serum concentrations of thyroid hormones (TH) and TSH showing high circulating TH levels along with an unsuppressed TSH represent the biochemical hallmark of RTHβ, as the expression of the impaired thyrotrope sensitivity to TH feedback. Interestingly, RTHβ patients exhibit inconstant manifestations and even patients harboring the same variant within one family may present with variable clinical phenotypes.

The clinical picture ranges from thyrotoxic manifestations to the absence of any signs of thyroid hormone excess. Differences in the degree of hormonal resistance are likely due to the variable TRβ and TRα expression in different tissues. Consequently, manifestations of TH deficiency and excess can coexist in one patient. As an example, hypercholesterolemia, delayed bone maturation, growth retardation, and learning disabilities (suggestive of hypothyroidism) may coexist with weight loss, osteoporosis, heat intolerance, hyperactivity, and tachycardia (typical of thyrotoxicosis). Here, we report a family with an unusual clinical presentation and biochemical findings of central hyperthyroidism that were initially attributed to interferences in thyroid function tests (TFTs).

Case Presentation

The index case of this family was a 30-year-old man presenting with hyperhidrosis and tachycardia. He had a positive family history for thyroid diseases since his mother underwent total thyroidectomy for a large goiter with compressive symptoms, while the younger brother had 2 thyroid nodules (<20 mm).

Initial investigations showed high fT4 and fT3 levels along with unsuppressed TSH (1.260 uIU/mL) assessed by Roche ELECSYS. TRAb was undetectable (0.14 IU/mL <1.0), and thyroid ultrasound revealed a gland volume of 17 mL with a slightly heterogeneous echostructure and a cystic lesion of 4 mm in the left lobe (Table 1). MRI revealed a partial empty sella without signs suggestive of microadenomas. Assessment of hypothalamic-pituitary axis showed a normal response to TRH test (TSH peak at 20 min = 11.00 uUI/mL) raising the suspect of RTHβ. The T3 suppression test was declined by the patient. At that time, relatives' TFTs measured by the Siemens CENTAUR platform and Ortho VITROS were within the normal range (Table 1).

THRB Gene Analysis and In Silico Analysis

Due to the suspect of RTHβ, a molecular analysis of the *THRB* gene was performed. Sanger direct sequencing technology revealed a novel variant in the exon 10 of *THRB* gene (NM_000461:

Table 1. Biochemical and clinical results of the family members according to the analytical method used to measure the TFTs

Patient	*THRB*	Year	TSH uUI/mL (0.4–5)	fT4 (lab-specific reference)	fT3 (lab-specific reference)	Method	Therapy	Thyroid US
I-1	wt	2020	0.76	1.02 ng/dL (0.78–1.94)	3.2 pg/mL (1.5–5)	Siemens	–	Normal
I-2	p.L428V	2018	0.95	1.60 ng/dL (0.78–1.94)	4.2 pg/mL (1.5–5)	Siemens	LT4 125 ug	2020: empty thyroid bed after total thyroidectomy
		2019	0.64	1.65 ng/dL (0.7–1.8)	4.3 pg/mL (2.2–4.2)	Siemens	LT4 125 ug	
		2020	5.2	22 pmol/L (12–22)	3.7 pmol/L (2–4.4)	Roche	LT4 125 × 5 + 100 × 2 days/week	
II-1	p.L428V	2018	1.26	26.80 pmol/L (12–22)	5.36 pg/mL (2–4.4)	Roche	–	2018 normal (17 mL, slightly heterogeneous echostructure)
		2019	1.4	27.7 pmol/L (9–20)	NA	DELFIA	–	
II-2	p.L428V	2013	0.76	15.3 pg/mL (7–17)	4.89 pg/mL (2.7–5.7)	Vitros	–	2013 nodular goiter (thyroid nodules 16 and 6 mm; stable during follow-up 2016–2020)
		2016	0.34	1.82 ng/dL (0.78–1.94)	–	Siemens	LT4 75 ug	
		2018	0.63	1.49 ng/dL (0.61–1.12)	–	Beckman	LT4 100 ug	
		2019	0.56	29.8 pmol/L (12–22)	3.97 pmol/L (2–4.4)	Roche	LT4 100 ug	
		2020	1.32	24.8 pmol/L (12–22)	4.4 pmol/L (2.4–4.4)	Roche	–	

US, ultrasonography; LT4, levothyroxine; NA, not assessed (FT3 kit no more commercially available); TFT, thyroid function test.

c.C1282G, p.L428V) not present in several data bases (NCBI dbSNP, Genome Browser, 1000 Genome, gnomAD, ExAC, and HGMD). The leucine 428 is located within the third hot spot region of TRβ and this residue is highly conserved across several species and in most of the nuclear receptors (data not shown), suggesting a possible pathogenic role of the variant.

In silico structural modeling (Fig. 1a) suggested that the substitution of a leucine by a valine residue with a smaller side chain may cause a structural perturbation by increasing the distance from neighboring hydrophobic residues. In silico analyses were performed by the wANNOVAR software (http://wannovar.wglab.org/). The wANNOVAR output results contain scores of several widely used prediction tools, including SIFT, PolyPhen2 HDIV, PolyPhen2 HVAR, LRT, MutationTaster, MutationAssessor, FATHMM, PROVEAN, MetaSVM, MetaLR, M-CAP, and fathmm-MKL. Eleven out of twelve prediction algorithms classified this variant as possibly pathogenic, in according to American College of Medical Genetics and Genomics standards and guidelines [5].

On the other hand, leucine 428 is involved in the dimerization of the TRβ with the RXR, and variants lacking the ability to form heterodimer are not expected to exert a dominant negative effect on the wild-type receptor. Indeed, the artificial substitution of the leucine with a polar charged (arginine) residue generates a receptor that cannot dimerize with RXR [6, 7]. Thus, also a naturally occurring variant at this codon could be expected to be silent [8].

Intriguingly, the same variant was found in the mother (I-2) and in the younger brother (II-2) who had an apparent normal thyroid function (Table 1; Fig. 1b), suggesting that the amino acid change had no impact on the receptor functions. Consequently, assay interference in the TFTs of the proband was initially assumed as the most likely explanation, being the hypothesis of a TSH secreting adenoma discarded due to results of MRI and dynamic testing [9].

Polyethylene Glycol Precipitation and Exclusion of Assays Interferences

To demonstrate the presence of an assay artifact, we first reassessed TFTs with a one-step assay (electrochemiluminescence immunoassay, Roche Elecsys, Mannheim, Germany) and with a two-step method (Wallac DELFIA Perkin-Elmer) both confirming the biochemical feature of central hyperthyroidism (Table 1). As proband's serum had been reported to us to potentially contain anti-T4 and anti-T3 antibodies on a radioimmunoprecipitation technique [10], we performed a polyethylene glycol (PEG) precipitation test, described by Beato-Víbora and Alejo-González [11], to exclude interference by circulating immunoglobulins on TFTs.

After PEG incubation of the proband's serum (1:1 w/w with a 25% PEG 6000 MW solution), TSH, fT4, and fT3 levels did not change significantly (recovery rate >40%), similar to what found in two control sera used (one hyperthyroid and one euthyroid, respectively). In contrast, PEG precipitation normalized free thyroid hormone levels in one patient affected with monoclonal gammopathy of undetermined significance with an IgM paraprotein, as well as in another patient with anti-T4 autoantibodies [11] (online suppl. Fig. 1a–c; see online Supplementary Materials).

Given the increased fT4/fT3 ratio measured after PEG precipitation in the index case (online suppl. Fig. 1b, c), we also sequenced the exon 7 of the albumin and the *TTR* gene, encoding for transthyretin to exclude a familial dysalbuminemic hyperthyroxinemia, and we did not find any variant. We did not sequence the *SERPINA7* as TT4 levels were increased (243.7 nmol/L, normal reference 69-141), thus excluding TBG deficiency. In addition, serum TBG levels were in the normal range (23 mg/L normal reference 13–39).

Additional Tests in Other Affected Family Members

Taken together, all these data were still highly suggestive for a mild form of RTHβ. Therefore, we performed additional investigations in the other family members harboring the same *THRB* variant and previously reported as unaffected. Interestingly, the

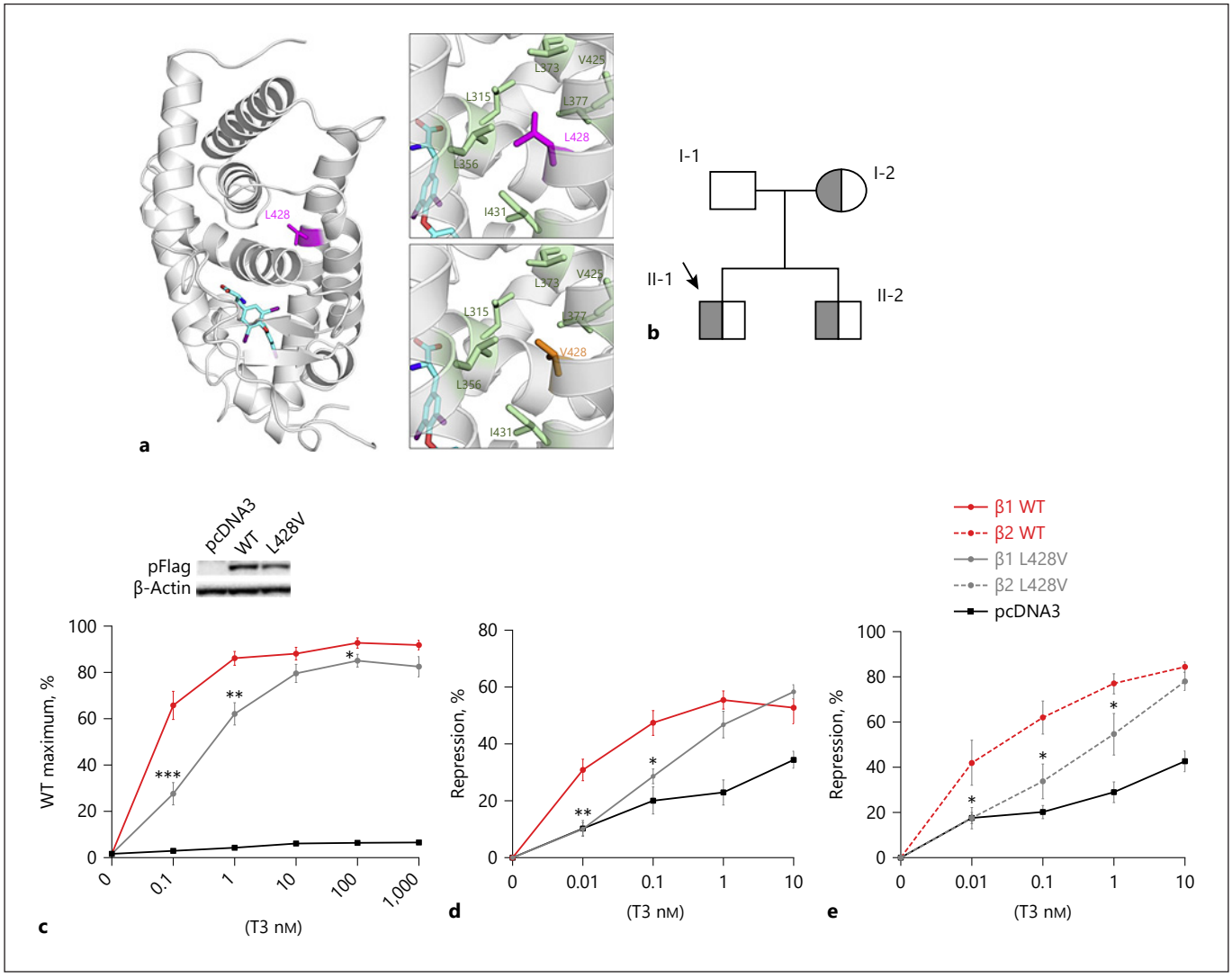


Fig. 1. Clinical and biochemical data of the family. **a** The crystal structure of human TRβ ligand binding domain bound to T3 (in cyan) is shown with leucine 428 (L428, magenta) located in H11 (PDB ID: 1BSX). Enlarged views show that L428 is part of a core of hydrophobic (green) residues. Substitution of this residue by valine (V428, orange), a conservative change to an amino acid with a smaller side chain increasing the distance from neighboring hydrophobic residues, might be expected to cause a small structural perturbation. **b** The family tree of the family shows the typical autosomal dominant inheritance of the *THRB* variant and its segregation with RTHβ. **c–e** Functional properties of L428V TRβ mutant. **c** Positive transcriptional regulation was assayed in JEG3 cells transfected with empty (pcDNA3), wild-type (β1WT), or mutant (β1L428V) receptors together with a reporter gene (MAL-TKLUC) and an internal control plasmid (BosβGal) and increasing T3 concentrations. Results are expressed as % of maximum WT receptor response and represent the mean ± sem of at least 4 independent experiments, each performed in triplicate. Inset shows western blot of flag epitope-tagged TR and control (β actin) proteins. **d**, **e** Negative transcriptional regulation was assayed in JEG3 cells transfected with empty (pcDNA3), WT, or mutant (L428V) receptors, tested in either TRβ1 (panel D) or TRβ2 (panel E) backgrounds together with the human, pituitary TSHα subunit reporter gene, and an internal control plasmid (BosβGal) and increasing T3 concentrations. Results are expressed as % repression of reporter gene activity relative to levels in cells cultured in the absence of T3 and represent the mean ± sem of at least 4 independent experiments, each performed in triplicate. Asterisks denote a statistically significant difference (*$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$) for WT and L428V mutant TRβ comparisons. WT, wild type; RTHβ, resistance to thyroid hormone β; TRβ, thyroid hormone receptor beta.

younger brother reported a euthyroid nodular goiter; thus, levothyroxine (LT4) suppressive treatment was given in the attempt to reduce nodular size. Although earlier investigations showed normal TFTs with appropriate fT4 levels according to the TSH levels, the administration of LT4 up to 100 μg failed to suppress the TSH (Table 1), despite the increasing fT4 levels, thus indicating a reduced thyrotrope sensitivity to exogenous LT4. In addition, when LT4 was discontinued and thyroid function measured by a different method (electrochemiluminescence immunoassay, Roche Elecsys, Mannheim, Germany), we disclosed the typical biochemical feature of central hyperthyroidism. Similar results were found in the mother I-2 (Table 1) who displayed high TSH despite fT4 levels at the upper limit of normal range after LT4 dose adjustment. This variability in fT4 and fT3 serum levels among patients harboring the same *THRB* variant is not surprising, and in online supplementary Table 1, we report 5 representative families in which fT4 and fT3 were highly variable ranging from −13 to +349% of the upper limit of the reference range.

Functional Characterization of the Mutant Receptor

To further support the pathogenicity of the variant, we performed several additional experiments. The results of the functional characterization of the mutant receptor are represented in Figure 1. The L248V variant showed a slightly reduced T3-induced transcriptional activity compared with wild-type using a reporter gene containing positively (malic enzyme, Panel C) regulated thyroid response element. At supraphysiological T3 concentrations, the L248V exhibited a similar maximal transcriptional activity as WT. When tested on a negatively regulated reporter gene (human TSH alpha subunit), we observed a significant impairment at low T3 concentrations, but maximal inhibition was achieved at higher T3 levels in either TRβ1 (panel D) or TRβ2 backgrounds (panel E).

Zebrafish Studies

The effects of L248V variant on thyroid functions were also assessed in vivo using zebrafish embryos as a model system, as previously done for human *THRA* variants [12]. Fifty pg/embryo of TRβ1-WT or TRβ1-L428V mRNAs was microinjected into zebrafish zygotes at 1–2 cell stage embryos. Compared with the TRβ1-WT, the regions of *tshba* and *tg* expression appeared significantly enlarged in TRβ1-L428V embryos at 2 and 4 days post-fertilization (dpf) (Fig. 2a–d, a′–d′, f, g). Consequently, the number of follicles producing T4 was increased in TRβ1-L428V larvae at 5 dpf (Fig. 2e–e′, h). To test the ability of T3 to suppress the high *tshba* levels of TRβ1-L428V model, the injected embryos were treated with growing doses of T3 (5–20 nM) from 6 h post-fertilization up to 2 dpf. The 5–10 nM T3 concentrations in the water were sufficient to completely suppress the *tshba* in TRβ1-WT control fish, whereas similar results were visible in TRβ1-L428V embryos at higher T3 doses (15–20 nM) (Fig. 2i–m, i′–m′, n).

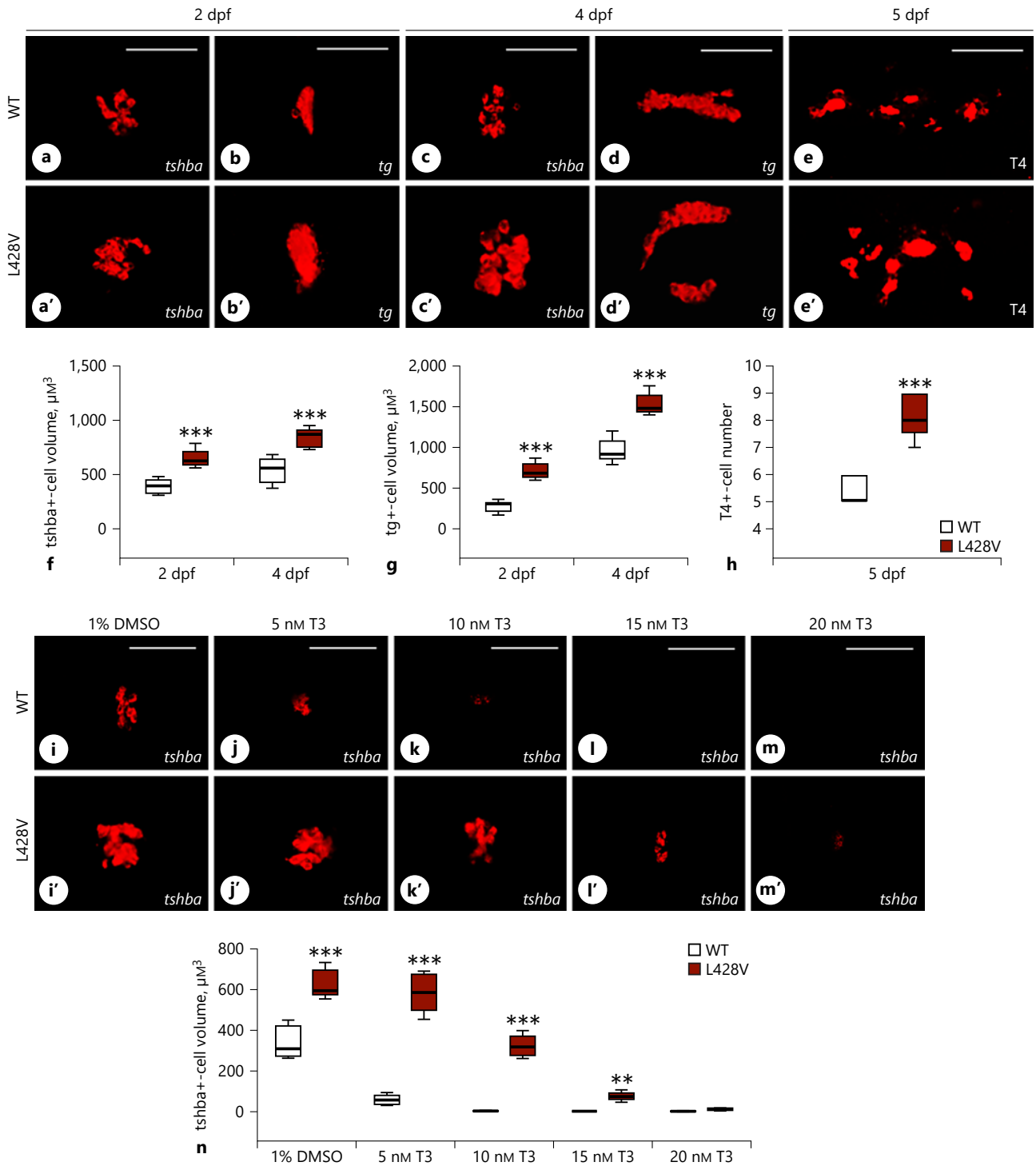
Discussion/Conclusion

We describe a family with RTHβ associated with multinodular goiter and mild thyrotoxic features with an unusual clinical presentation. In particular, the variant was initially misinterpreted as not pathogenic because it is not apparently segregating with the phenotype. Nonetheless, the lack of TSH suppression with pharmacological dose of levothyroxine in the proband's brother provided the clue to uncover the proper diagnosis of RTHβ in all the relatives harboring the p.L428V variant.

In this family, the correct diagnosis of RTHβ was hampered by several factors. The main source of uncertainty was the conflicting results of TFTs in two affected individuals, who were revealed to actually have high fT3 and fT4 levels only when measured by alternative one-step immunoassays such as Roche and Beckman and one two-step method (Wallac DELFIA Perkin-Elmer), while normal TFTs were observed on Siemens and Vitros platforms. It is generally believed that, in patients with suspect of central hyperthyroidism, the presence of inconsistent results between different assay methods should be attributed to spurious hyperthyroxinemia due to serum interference [13]. Conversely, to the best of our knowledge, an underestimation of fT3 and fT4 in patients with a genuine central hyperthyroidism has never been described so far.

Although in silico analysis and the evaluation of a computational model of the mutant receptor were both suggestive of a pathogenic role of the p.L428V variant, it is well known that the prediction algorithms are not always fully reliable for the evaluation of variants with unknown significance [14, 15]. In addition, previous functional studies have shown that an artificial variant at the same codon (R428L) lack of a dominant negative effect on wild-type receptor due to its inability to dimerize with RXR. Therefore, one could predict that also naturally occurring variants of this residue would not be associated with RTHβ [6–8]. Consequently, the discrepant TFTs were thought to be attributable to the possible presence of interference and the p.L428V variant was considered a variant with unknown significance not responsible for the proband's phenotype.

Nevertheless, the PEG precipitation test demonstrated that in patient II-1 the biochemical signature was genuine. This observation made additional investigations necessary, which allow us to finally uncover the RTHβ diagnosis in 3 patients. The results of the in vitro studies were suggestive of a slightly impaired transcriptional regulation of either negative or positive regulated reporter genes, which was rescued with supra-physiological doses of T3. This mild impairment was congruent with the modest elevation of serum fT4 and fT3, which was evident only in few assay platforms. Moreover, the RTHβ was missed at the baseline assessment in the two patients with lower thyroid hormone levels, but became evident



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after the administration of exogenous levothyroxine in the attempt to suppress the TSH or to correct an iatrogenic hypothyroidism.

Importantly, the in vivo zebrafish model rather than the in vitro artificial one clearly revealed the potential dominant negative action of the p.L428V variant, as the TRβ1-L428V zebrafish zygotes displayed an increased expression of tshba and tg and a higher number of T4-positive follicles. Moreover, the administration of growing doses of T3 (5–20 nM) indicated the reduced ability of T3 to suppress the high tshba levels in the TRβ1-L428V fish.

The reason why the patient's mother and brother had inconsistent TFTs is unclear, but it is very unlikely that they both had an assay interference causing an underestimation of free thyroid hormone levels. It is possible that some analytical methods (Siemens CENTAUR and Ortho VITROS) may have failed to reveal central hyperthyroidism because I-2 and II-2 had only slightly elevated TH. Indeed, although several attempts to harmonize thyroid hormone assays, the evidence collected over the past 2 decades suggests that a significant inaccuracy of fT4 and fT3 measured by several immunoassays still exists [16].

The degree of such variability is not negligible, as Giovannini et al. [17] found 20% bias values compared to the consensus mean, in several one- and two-step assays. Similarly, Steele et al. [18] found that more than 50% of results were unacceptably variable in 13–60% of assays evaluated in that study. The inaccuracy of TFTs, coupled with dissimilar reference intervals (RI), complicates test interpretation. The implementation of evidence-based harmonized RI is a critical step toward accurate result interpretation and optimized patient care. As shown in Table 1, despite the attempt of harmonized TFTs, differences in RI persist between laboratories even when using the same platforms and the same reagents [19].

As an example, in patient II-2 such imprecision could easily explain the normal fT4 values measured by some immunoassay (correct value = 24 pmol/L – 20% = 19.2 pmol/L) (Table 1). This may be attributed to the different levels/affinity of serum binding proteins (TBG, transthyretin, and albumin), competing drugs, heterophilic antibodies, age, and medical conditions such as end-stage renal disease, major cardiac surgery, and critical illness [20–23]. In fact, a better consistency may be found by ultrafiltration LC-MS/MS, which is less influenced by the transport proteins [16].

Interestingly, in this family there was a large variability in the clinical manifestations of RTHβ, being the proband more severely affected compared to his brother. In the patient's brother (II-2), the impaired sensitivity to TH feedback has been disclosed only during the attempt of TSH suppression with LT4 for nodular goiter. This variability both in TH levels and in the clinical phenotype among patients harboring the same *THRB* variant has been previously reported [24] and probably more common than what reported in the literature. Indeed, we observed very divergent TH levels also in other 5 families of our monocentric RTHβ cohort (online suppl. Table 1). This high variability in TFTs in RTHβ has been shown also by Dieu et al. [23] in a cohort of 105 RTHβ patients, including 38 cases harboring the 5 more common variants of this cohort. They found that 89% of patients had increased fT4 and fT3 levels, while an isolate increase of fT4 or fT3 was observed in 8 and 3% of cases, respectively.

These findings are not surprising given the interindividual differences in the setpoint of the hypothalamic-pituitary-thyroid axis, which is observed also in healthy subjects, as a result of a complex polygenic basis [25]. Other variants located close to the end of the "cold region" of the receptor show discrepancy between fT4 and fT3 serum levels and the degree of T3 binding impairment. As an example, the R429Q variant was associated with elevated fT4/fT3 levels, despite a normal Ka for T3, while the I431T variant was associated with a mild increase in fT4/fT3 although a severe impairment of T3 binding. In addition, the clinical expression and free thy-

Fig. 2. Zebrafish studies. Microinjection of β1WT or β1L428V transcripts (50 pg/embryo) into zebrafish zygotes at 1–2 cell stage. ISH of tshba and tg probes and confocal acquisition of fluorescent signals emitted by FastBlue dye at 2, 4 and 5 dpf (**a–e**, **a′–e′**). Immunofluorescence of T4+ follicles using anti-T4 primary antibody (rabbit anti-T4 polyclonal antibody (1:1,000; MP Biochemicals) and Alexa Fluor 555 (1:500; Thermo Fisher Scientific). Embryos were acquired in the ventral view, anterior to the top. Scale bars: 50 μm. For quantification of WT and L428V effects on thyroid function, the cell volume (μm^3) of *tshba* or *tg* signals was calculated with the ImageJ software (**f**, **g**) or by counting the number of T4+ follicles (**h**). The ability of T3 to suppress the tshba levels in WT and L428V embryos at 2 dpf was analyzed treating the embryos from 6 hpf with growing doses of T3 (Sigma, 5–20 nM) followed by ISH of *tshba* and confocal analysis of fluorescent signals (**i–m**, **i′–m′**, **n**) as described in the figure. For each experiment, 30 embryos derived from 3 independent injections were analyzed. Asterisks indicate statistically significant differences (Student's *t* test; $^{**}p < 01$; $^{***}p < 001$) of L428V embryos versus WT controls. ISH, in situ hybridization; WT, wild type; dpf, days post-fertilization; hpf, hours post-fertilization.

roid hormones levels are often variable among kindred harboring the same variants located in this region [26].

In conclusion, the variable results of TFTs on different immunoassays and the flawed report of interference by anti-T4/T3 antibodies have been a source of diagnostic delay, despite the finding of a novel *THRB* gene variant in this family. The dominant inheritance of the biochemical hallmark of RTHβ (unsuppressed TSH despite TH levels at or above the upper limit of normal) co-segregating with the p.L428V variant gave support to its pathogenic role, thus expanding the spectrum of *THRB* gene variants causing RTHβ. Both in vitro and in vivo studies gave definitive evidence of the mild functional alteration of the mutant TRβ, but the in vivo zebrafish assay was the only one able to reveal the dominant negative action of the p.L428V variant.

In the absence of a reliable biomarker of peripheral TH actions, we suggest that the familial screening of patients with suspected RTHβ should be performed with a robust assay such as liquid chromatography-tandem mass spectrometry or by the combination of more than one immunoassay. This is indicated not only in case of discrepancy among the segregation of a certain genetic variant and the biochemical data but also to select familial cases requiring molecular analysis of the *THRB* gene.

Statement of Ethics

The study was approved by the Ethics Committee of IRCCS, Istituto Auxologico Italiano (study protocol "CASI" reference no. 02C502_2005). Written informed consent was obtained from the patient for publication of this case report and any accompanying images.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

All authors contributed to the article and approved the submitted version. I.C. designed the study and wrote the draft of the manuscript. T.D.F., G.R., and O.R. performed the molecular analyses. B.R.A. and M.A[b]. performed the in silico molecular modeling. M.A[b]. also performed the molecular in vitro characterization. F.M. and I.G. performed the zebrafish studies. S.F., F.F., and S.C. collected the clinical data. M.A[f]. performed the serological assays and the P.E.G. precipitation studies. L.P., L.F., and K.C. reviewed and revised the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material files. Further enquiries can be directed to the corresponding author.

References

1 Refetoff S, Bassett JH, Beck-Peccoz P, Bernal J, Brent G, Chatterjee K, et al. Classification and proposed nomenclature for inherited defects of thyroid hormone action, cell transport, and metabolism. Thyroid. 2014 Mar;24(3):407–9.

2 Achermann JC, Schwabe J, Fairall L, Chatterjee K. Genetic disorders of nuclear receptors. J Clin Invest. 2017 Apr;127(4):1181–92.

3 Persani L, Campi I. Syndromes of Resistance to Thyroid Hormone Action. Exp Suppl. 2019;111:55–84.

4 Collingwood TN, Wagner R, Matthews CH, Clifton-Bligh RJ, Gurnell M, Rajanayagam O, et al. A role for helix 3 of the TRbeta ligand-binding domain in coactivator recruitment identified by characterization of a third cluster of mutations in resistance to thyroid hormone. EMBO J. 1998 Aug;17(16):4760–70.

5 Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American college of medical genetics and genomics and the association for molecular pathology. Genet Med. 2015 May;17(5):405–24.

6 Collingwood TN, Adams M, Tone Y, Chatterjee VK. Spectrum of transcriptional, dimerization, and dominant negative properties of twenty different mutant thyroid hormone beta-receptors in thyroid hormone resistance syndrome. Mol Endocrinol. 1994 Sep;8(9):1262–77.

7 Nagaya T, Jameson JL. Distinct dimerization domains provide antagonist pathways for thyroid hormone receptor action. J Biol Chem. 1993 Nov 15;268(32):24278–82.

8 Hayashi Y, Sunthornthepvarakul T, Refetoff S. Mutations of CpG dinucleotides located in the triiodothyronine (T3)-binding domain of the thyroid hormone receptor (TR) beta gene that appears to be devoid of natural mutations may not be detected because they are unlikely to produce the clinical phenotype of resistance to thyroid hormone. J Clin Invest. 1994;94:607–15.

9 Campi I, Covelli D, Moran C, Fugazzola L, Cacciatore C, Orlandi F, et al. The differential diagnosis of discrepant thyroid function tests: insistent pitfalls and updated flow-chart based on a long-standing experience. Front Endocrinol. 2020 Jul;11:432.

10 Trimarchi F, Benvenga S, Costante G, Barbera C, Melluso R, Marcocci C, et al. Identification and characterization of circulating thyroid hormone autoantibodies in thyroid diseases, in autoimmune non thyroid illnesses and in lymphoreticular system disorders. J Endocrinol Invest. 1983 Jun;6(3):203–9.
11 Beato-Víbora PI, Alejo-González S. Avoiding misdiagnosis due to antibody interference with serum free thyroxin. Int J Endocrinol Metab. 2016 Nov;15(1):e37792.
12 Marelli F, Carra S, Rurale G, Cotelli F, Persani L. In vivo functional consequences of human THRA variants expressed in the zebrafish. Thyroid. 2017;27(2):279–91.
13 Dieu X, Sueur G, Moal V, Boux de Casson F, Bouzamondo N, Bouhours N, et al. Apparent resistance to thyroid hormones: from biological interference to genetics. Ann Endocrinol. 2019 Nov;80(5-6):280–5.
14 Pappa T, Refetoff S. Resistance to thyroid hormone beta: a focused review. Front Endocrinol. 2021 Mar 31;12:656551.
15 Korwutthikulrangsri M, Dosiou C, Dumitrescu AM, Refetoff S. A novel G385E variant in the cold region of the T3-binding domain of thyroid hormone receptor beta gene and investigations to assess its clinical significance. Eur Thyroid J. 2019 Dec;8(6):293–7.
16 Araque KA, Klubo-Gwiezdzinska J, Nieman LK, Welsh K, Soldin SJ. Assessment of thyroid function tests and harmonization: opinion on thyroid hormone harmonization. Ther Adv Endocrinol Metab. 2019 Dec; 10: 2042018819897049.
17 Giovannini S, Zucchelli GC, Iervasi G, Iervasi A, Chiesa MR, Mercuri A, et al. Multicentre comparison of free thyroid hormones immunoassays: the Immunocheck study. Clin Chem Lab Med. 2011 Oct;49(10):1669–76.
18 Steele BW, Wang E, Klee GG, Thienpont LM, Soldin SJ, Sokoll LJ, et al. Analytic bias of thyroid function tests: analysis of a college of American pathologists fresh frozen serum pool by 3900 clinical laboratories. Arch Pathol Lab Med. 2005;129(3):310–7.
19 Parker ML, Adeli K; CSCC Working Group on Reference Interval Harmonization. Pediatric and adult reference interval harmonization in Canada: an update. Clin Chem Lab Med. 2018 Dec 19;57(1):57–60.
20 Després N, Grant AM. Antibody interference in thyroid assays: a potential for clinical misinformation. Clin Chem. 1998 Mar;44(3): 440–54.
21 Schoenmakers N, Moran C, Campi I, Agostini M, Bacon O, Rajanayagam O, et al. A novel albumin gene mutation (R222I) in familial dysalbuminemic hyperthyroxinemia. J Clin Endocrinol Metab. 2014 Jul;99(7):E1381–6.
22 Koulouri O, Moran C, Halsall D, Chatterjee K, Gurnell M. Pitfalls in the measurement and interpretation of thyroid function tests. Best Pract Res Clin Endocrinol Metab. 2013 Dec; 27(6):745–62.
23 Dieu X, Bouzamondo N, Briet C, Illouz F, Moal V, Boux de Casson F, et al. Familial dysalbuminemic hyperthyroxinemia: an underdiagnosed entity. J Clin Med. 2020 Jul 3;9(7): 2105.
24 Fujisawa H, Gagné J, Dumitrescu AM, Refetoff S. Very severe resistance to thyroid hormone β in one of three affected members of a family with a novel mutation in the THRB gene. Thyroid. 2019 Oct;29(10):1518–20.
25 Medici M, Visser WE, Visser TJ, Peeters RP. Genetic determination of the hypothalamic-pituitary-thyroid axis: where do we stand? Endocr Rev. 2015 Apr;36(2):214–44.
26 Hayashi Y, Weiss RE, Sarne DH, Yen PM, Sunthornthepvarakul T, Marcocci C, et al. Do clinical manifestations of resistance to thyroid hormone correlate with the functional alteration of the corresponding mutant thyroid hormone-beta receptors? J Clin Endocrinol Metab. 1995 Nov;80(11):3246–56.