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Version of Record: https://www.sciencedirect.com/science/article/pii/S0003426619300915
Manuscript_6be91f94a3759ffb8a178ad8e318a1a2

Apparent resistance to thyroid hormones: from biological interference to genetics
Résistance présumée aux hormones thyroïdiennes : de l'interférence biologique à la génétique

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Abstract
Resistance to thyroid hormones syndrome is defined as increased thyroxine (T4) and triiodothyronine (T3) concentrations associated with normal or sometimes increased thyrotropin (TSH) concentration. This is usually due to a pathogenic variant with loss of function of the gene coding for thyroid hormone receptor β (*THRB*). This discrepancy in thyroid hormones (TH) and TSH concentrations is also frequently observed in the presence of analytical interference, notably alteration of TH transport proteins in serum.
During 2017, 58 samples were sent to our laboratory in the Angers University Hospital Rare Thyroid and Hormone Receptor Disease Reference Center in order to identify an etiology for discrepant TSH and TH results. We sequenced the genes involved in TH regulation, action and transport *(THRB, THRA, SECISBP2, SLC16A, ALB, TTR, SERPINA7)*. Free T4 and free T3 assay were performed with a second immunoassay (Siemens ADVIA Centaur).
A genetic cause of discrepancy in TH and TSH concentrations, with mutation in *THRB*, was found in 26% of cases (15/58). Biological interference due to TH serum transport protein variant was found in 24% (14/58) of cases. No pathogenic variants were found in the other genes studied. Biological interference was also suspected in 26% of cases without genetic variant, in which the biological discrepancy was not confirmed by a second analytical technique (15/58). Finally, no etiology for the biological discrepancy could be found in 24% of cases (14/58). Clinically, patients in whom biological discrepancy was due to analytic interference were more often asymptomatic, and patients with no identified etiology tended to be older.
To limit diagnostic errors associated with the finding of discrepant TSH and TH, we recommend initially conducting a second thyroid function test (TSH, free T4 and free T3) with a different assay, and then screening for a genetic variant in gene coding for thyroid hormone receptor β (*THRB)* and the TH serum transport proteins (*ALB, TTR, SERPINA7*).

Résumé
Le syndrome de résistance aux hormones thyroïdiennes est défini comme une augmentation des concentrations de thyroxine (T4) et de triiodothyronine (T3) associée à une concentration normale ou élevée de thyrotropine (TSH). Le plus souvent, ce syndrome est dû à un variant pathogène avec perte de fonction du gène codant pour le récepteur bêta aux hormones thyroïdiennes (*THRB*). Cette discordance entre les concentrations d'hormones thyroïdiennes (HT) et de TSH est également observée en présence d'interférences analytiques, en particulier lors de la modification des protéines de transport des HT dans le sérum.
Au cours de l'année 2017, 58 échantillons ont été adressés au Centre de référence des maladies rares de la thyroïde et des récepteurs hormonaux du CHU d'Angers afin de déterminer l'étiologie de résultats discordants entre TSH et HT. Nous avons réalisé une analyse génétique pour les principaux gènes impliqués dans la régulation, action et transport des HT *(THRB, THRA, SECISBP2, SLC16A, ALB, TTR, SERPINA7)* et une quantification de T4 libre et T3 libre avec une deuxième technique d'immunoanalyse (Siemens ADVIA Centaur).
Parmi les 58 échantillons, un syndrome de résistance aux hormones thyroïdiennes, avec mutation du gène *THRB*, a été retrouvé dans 26 % des cas (15/58). Une interférence biologique due à un variant des protéines de transport des HT a été constatée dans 24 % des cas (14/58). Aucune mutation n'a été trouvée dans les autres gènes étudiés. Dans 26 % des cas, la discordance biologique entre le dosage

des HT et de la TSH n'avait pas été confirmée par une seconde technique d'analyse, faisant suspecter une interférence biologique (15/58). Enfin, nous n'avons retrouvé aucune étiologie à la discordance biologique dans 24 % des cas (14/58). Cliniquement, les patients dont la discordance biologique était liée à une interférence analytique étaient le plus souvent asymptomatiques et les patients sans étiologie retrouvée étaient plus âgés.
Afin de limiter les erreurs de diagnostic associées à la découverte de concentrations de TSH et d'HT discordantes, on peut recommander de commencer par effectuer une seconde évaluation de la fonction thyroïdienne (TSH, T4 libre et T3 libre) avec une technique différente, puis de rechercher un variant génétique dans le gène codant pour le récepteur bêta aux hormones thyroïdiennes (*THRB*) et les protéines de transport TH dans le sérum (*ALB*, *TTR*, *SERPINA7*).

Keywords: resistance to thyroid hormones; immunoassay interference; *THRB*; transthyretin; albumin; *TBG*
Mots-clés : résistance aux hormones thyroïdiennes ; interférence analytique ; *THRB* ; transthyrétine ; albumine ; TBG

Abbreviations : RTH : resistance to thyroid hormone, TH : thyroid hormone(s), TTR : transthyretin, TBG : thyroxine binding globulin, TFT : thyroid function test(s), fT4 : free thyroxine, fT3 : free triiodothyronine.

1. Introduction

First described by Refetoff et al in 1967 [1], the resistance to thyroid hormone syndrome (RTH) is a rare disease (1 case in every 50,000 births). Clinical manifestations are variated. The most frequent are a goiter (85%), tachycardia (75%) and attention deficit hyperactivity disorder (70% of children). These non-specific symptoms may lead to thyroid hormone assessment and to the detection of dissociated results for TFT (thyroid function test i.e. increase in thyroid hormone (TH) and a normal or mildly elevated concentration of TSH).

In 1989, the phenotype was related to mutations in the gene *THRB* coding for thyroid receptor β (TRβ). Indeed, thyroid hormones exert their biological actions via two types of thyroid receptor: TRβ

and TRα. Their distribution and expression level vary among tissues [2]. TRβ is expressed in the hypothalamus and pituitary gland where it conveys the TH negative feedback on TSH secretion. In case of an inactive TRβ caused by a pathogenic variant in *THRB*, the TH concentration needed in order to regulate the TSH secretion is higher than in normal individuals. This explains the biological phenotype: high levels of T4 and T3, and unsuppressed TSH. Moreover, the high levels of T4 and T3 explain the clinical manifestation of the RTH, through TH action mediated by functional TRα especially in heart, gut and autonomous nervous system.

Other genes with modified TH action are involved in the discrepancy between TH and TSH concentrations [3]. We can find genes coding for thyroid receptor α (*THRA*) [4], for the protein involved in insertion of selenocystein in deiodinases (*SECISBP2*) [5], and for the hormone cell membrane transporter MCT8 (*SLC16A2*) [6]. Thyrotropic pituitary adenoma may produce the same hormonal phenotype. The diagnosis will be confirmed by the identification of a pituitary tumor via MRI. However the most frequent differential diagnosis of RTH are assay artefacts. Assessment of TH and TSH concentration mainly relies on immunoanalysis techniques. The TH circulating in the blood is mainly bound to proteins, whereas a very small fraction (0.02% and 0.3 % for T4 and T3, respectively) is free [7]. The three main binding proteins are albumin, transthyretin (TTR) and thyroxin-binding globulin (TBG). These proteins bind T3 and T4 in different proportions. The assessment of free T4 (fT4) and free T3 (fT3) by immunoanalysis techniques may therefore be influenced by various factors. It is essential to note that the impact of these factors is directly related to the type of immunoanalysis platforms used [8, 9]. Main types of interference in TFT are biotin, and antibodies (antistreptavidin antibodies, antirhutenium TH antibodies and heterophilic antibodies) [10, 11, 12]. Other factors may affect by the equilibrium between free and bound TH, such as heparin, non-steroidal anti-inflammatory drugs, antibodies as well as quantitative and qualitative changes in the binding proteins [9] .

The latter constitute the field of this study. Genetic variants affecting the affinity of these three TH transport proteins (Albumine, TTR, TBG) for the TH have been reported. The most frequent involve albumin, leading to familial hyperthyroxinemic dysalbuminemia, with a prevalence of 0.01 to 1.8 %,

depending on the population studied. Mutations are located in the T4 binding site (codon 218 and 221) and enhance affinity [13]. The transthyretin variants are sorted into amyloidogenic and non-amyloidogenic. The most frequent variant involves codon 109 and is non-amyloidogenic [7]. The TBG variants lead to partial or total deficit of the protein and to low total TH concentration whereas the free TH concentration is usually normal [14]. In all these situations, the change of affinity for TH modifies the equilibrium between free and bound hormones in the immunoassay. These variants have no thyroid-related clinical consequence for the patient, but identifying these artefacts is of importance to avoid unnecessary therapeutics.

Our Reference Centre for Rare Diseases of Thyroid and Hormone Receptors, receives around 50 samples each year for *THRB* sequencing because of dissociated thyroid function tests (TFT). Only a quarter of these cases exhibit a mutation of *THRB*. We therefore tried to explain the very high proportion of cases with no mutation despite a suspicion of RTH syndrome based on discordant TFT.

2. Patients and Methods

2.1 Patients

Fifty-eight patients (22 male and 36 female), investigated during year 2017 for suspicion of RTH syndrome, were included in the study. All patients came from several regions of France. All had either free T4 (fT4) and / or free T3 (fT3) concentration above the upper limit of the normal range of the assay used in their routine laboratory with normal or elevated TSH concentration. Clinical data were collected from referring doctor of patients. Cardiac signs were defined by a supraventricular arrythmia (flutter or fibrillation), a sinusal tachycardia, other conduction abnormalities or a cardiac failure. The hyperactivity, agitation or attention deficit were reported by the patient or his family. Ultrasonography of thyroid was performed to detect goiter or thyroid nodules.

2.2 Genetic analysis

All patients signed an informed consent form for genetic testing. DNA was extracted from all blood samples with an EZ1 extraction kit (Qiagen, Hilden Germany). High throughput sequencing of the

THRB, ALB, TTR, SERPINA7, THRA, SLC16A2, and SECISBP2 genes were performed on an Ion proton sequencer (Thermofischer scientific, Waltham, USA).

2.3 Thyroid function tests

The first thyroid function tests were performed in different laboratories. Information on technique, assay used and normal ranges were available for 46/58 patients. Secondarily, we repeated fT4 and fT3 analyses with another assay in our laboratory on a Siemens ADVIA Centaur for 49 plasma samples. Normal ranges were for fT4: 9-19 pmol/L and for fT3: 2.6-5.7 pmol/L).

2.4 Statistics

Statistical analysis was performed with XLSTAT® and BiostaTGV. A Mann Whitney test was used for quantitative data and a Fischer Exact Test for clinical qualitative data. Results were expressed as median +/- Standard Deviation for age. Plots were made using Microsoft Excel or using Plotly (through its Python API).

3. Results

3.1 Genetic analysis

Genetic analysis was performed for the 58 samples [FIGURE 1]. A pathogenic variant with loss of function in *THRB* was found in 15/58 cases (26%) confirming the diagnosis of RTH. Variants were found in genes coding for TH serum transport proteins in 14 cases, 8 in *ALB* (13.8%) and 6 in *TTR* (10.3%) and 0 in *SERPINA7*. No pathogenic variant was identified in *THRA, SECISBP2, SLC16A2*.

3.2 Thyroid function test verification

We quantified fT3 and fT4 for 49 samples with the same immunoassay (Siemens ADVIA Centaur) [TABLE 1].

Out of 10 plasma samples available from 15 patients with a *THRB* mutation, 9 displayed both an elevated fT4 and fT3 concentration, while only one displayed an isolated increase fT3. There was no difference in the extent of increase between the different pathogenic variants.

Thirteen out of 14 plasma samples taken from patients with genetic variant of TH serum transport proteins could be assessed. For the 8 patients with albumin variant, 3 had normal fT4 and fT3 concentration, 3 had both elevated fT4 and fT3, 2 had an increase in fT4 and none had an increase in fT3 alone. For the 6 patients with TTR variant, 2 had normal fT4 and fT3 concentration, none had both elevated fT4 and fT3, 1 had an increase in fT4 and two had an increase in fT3 alone.

Twenty-six samples were available for 29 patients without any genetic variant identified. In 15 out of 26, normal fT4 and fT3 were found, which suggests an analytical interference in the assay initially used in the referring centre. In 11 samples, a discrepancy between TH and TSH values was confirmed with both elevated fT4 and fT3 for 5 samples, an increase in fT4 alone for 3 samples and an increase in fT3 alone for 3 samples.

3.3 Clinical Information

We subdivided the patients into 3 groups: the first group, a RTH group (N=15) of patients with a pathogenic variant of *THRB*; the second group, an Analytical interference group (N= 29) of patients with genetic variants of TH serum transport proteins and patients with a normal biological assay with a second analytical technique; and the third group, an unexplained discrepancy group (N=14) of patients with abnormal biological assay (fT3, fT4) even using two different techniques or patients with no second hormonal assay.

The mean age of patients was 36 years (19 months to 78 years). The patients in unexplained discrepancy group were older than patients in RTH group (50+/-22 versus 29 +/-16, p= 0.011) or analytical interference group (30 +/-22, p= 0.072). Sixty percent (9/15) of patients in RTH group were diagnosed before the age of 30 and 100% before the age of 60. Twenty-nine percent (4/14) of patients in unexplained discrepancy group were diagnosed before the age of 30 and 36 % (5/14) after the age of 60. An analytical interference was identified more often in women (19/29) after the age of 30 in 63% (12/19) of them. At the opposite an analytical interference were rarer in men (10/29) and was found in 80% (8/10) of them before 30. A fisher exact test showed that this result was statistically significant with a p-value of 0.050 [FIGURE 2].

Clinical data were available in 83% of patients (48/58). These clinical informations revealed that the findings of discrepant thyroid functional testing were fortuitous in 50% of analytical interference, in 20% of RTH and 15% of unexplained discrepancy (p= 0.072) [FIGURE 3B].

Cardiac symptoms and hyperactivity were identical and not statistically different between three groups of patients. Cardiac symptoms, tachycardia or atrial fibrillation, were reported in 30% of patients in the RTH group, 35% in the analytical interference group and 38% in the unexplained discrepancy group. Hyperactivity reported by the patient or his family was found in 10% of the RTH patients, 15% in the analytical interference group and 23% in the unexplained discrepancy group.

A non-significant trend for a lower prevalence of goiter was observed in the analytical interference group, lower than both the confirmed RTH group and the unexplained discrepancy group: 15% versus 40% and 38%, respectively (p=0.149).

4. Discussion

We report the biological findings from 58 suspected cases of RTH submitted to the Reference Centre for Rare Diseases of Thyroid and Hormone Receptors during 2017. Fifteen mutations of *THRB* were identified, confirming the suspected diagnosis, along with 14 variants of TH serum proteins transport (*ALB* and *TTR*) producing analytical interferences. The quantification of fT3 and fT4 using another technique than the initial one confirmed increased fT4 and/or fT3 in 100% cases of RTH, in 61% of patients with TH protein transport variants and in 42% of unexplained discrepancies.

In our series, at first glance, only 26% of suspected cases of RTH are confirmed, which may appear very low compared to the literature [3]. When we looked for other genes which may produce a discrepancy in thyroid test with various mechanisms, we found no other genetic defects that reduce the TH biological activity.

We therefore looked for artefacts that may produce the same discrepancy in a thyroid test. Since biological interferences are numerous and vary between different assays, we measured TH again in an assay different from the initial one. Alternatively normal and abnormal results for the same patients, depending of the method used, strongly suggest a technique-related artefact [16, 17]. The reference technique associating equilibrium dialysis with subsequent TH assay in mass spectrometry is rarely used as it is technically demanding [18]. The verification of fT3 and fT4 was performed on a Siemens Advia Centaur automate. This immunoassay does not suppress all the interferences and can also be disturbed by artefacts. Nevertheless, the simple verification in a different assay allows for the apparent normalisation of the thyroid test, which suggests the presence of interferences, in 26% of cases.

Among possible causes of erroneous thyroid test, the presence of anti-T4 or anti-T3 antibodies, Heterophilic antibodies (HAMA), anti-avidin antibodies are well known, not forgetting the biotin interference in streptavidine-biotin based assays. However, genetic variants modifying the affinity of serum transport proteins for TH may also disturb the equilibrium between free and bound hormones and impair the performance of free hormone assays. Here we found variants of albumin and

transthyretin in 24% of cases. It should be stressed that this is as frequent as mutations of *THRB* in this specific population. Noteworthy, the analytical interferences due to serum transport protein variants are assay-specific, as was previously mentioned for familial dysalbuminemic hyperthyroxinemia [19, 20].

This study allowed us to reduce the group of true unexplained discrepant thyroid test, i.e. absence of mutation of *THRB*, or transport protein and supposed analytical interference to 24% of cases. This is in agreement with the reported frequency (15%) of RTH with no *THRB* mutation. Also, we confirm that in patients with high fT4 and fT3 and non-suppressed TSH, in absence of mutation of *THRB* there is very little chance of finding a mutation in the other genes involved in syndromes of reduced sensitivity to thyroid hormone, up to now.

Therefore, to avoid unreliable diagnoses, initially ruling out analytical interferences by simply repeating the thyroid test in different assays is mandatory. Genetic analysis of *THRB,* and TH serum transport proteins, should then be performed. Ultimately, other genes of the thyroid hormone signalling pathway could be analysed although we did not find any pathogenic variant in the latter. Although new generation sequencing techniques allow for an easy sequencing of many genes in the same run, they should not substitute a cautious step by step analysis of cases.

FUNDING

Grant from Angers University Hospital and National Research Agency THYROMUT

Conflict of Interest: none declared

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Table 1 : Quantification of ft4 and fT3 realised on a Siemens ADVIA Centaur according to the etiology of the TSH/TH discrepancy.

	Genetic variants (N=29)			No genetic variant (N=29)
	THRB (N=15)	*ALB* (N=8)	*TTR* (N=6)	
fT4 Nl fT3 Nl	0	3	2	15
fT4 ↗ fT3 ↗	9	3	0	5
fT4 ↗ fT3 Nl	0	2	1	3
fT4 Nl fT3 ↗	1	0	2	3
No data	5	0	1	3

Nl : Quantification within the limits of normality (fT4: 9-19pmol / l; fT3: 2.6-5.7pmol / l)

↗ : Quantification superior to the upper limit of normality.

N: Sample number

Figure 1 : Etiology of the TSH/TH discrepancy according to the presence or absence of genetic variant for 58 samples sent in 2017 to the University Hospital of Angers. Sequencing of *THRB* (Thyroid Hormone Receptor β), *THRA* (Thyroid Hormone Receptor α), *SLC16A2* (Solute Carrier Family 16 Member 2), *SECISBP2* (Selenocysteine insertion sequence binding protein 2), *ALB* (Albumin), *TTR* (Transthyretin)) and *SERPINA7* (thyroxin binding globulin (TBG)) was performed for the 58 samples. fT4 and fT3 quantification was performed on samples at the University hospital of Angers on a Siemens ADVIA Centaur. **Nl**: Quantification within the limits of normality (fT4: 9-19pmol / l; fT3: 2.6-5.7pmol / l) RTH : Resistance to thyroid hormone due to a variant of *THRB*, TH: thyroid hormones; N: number of cases

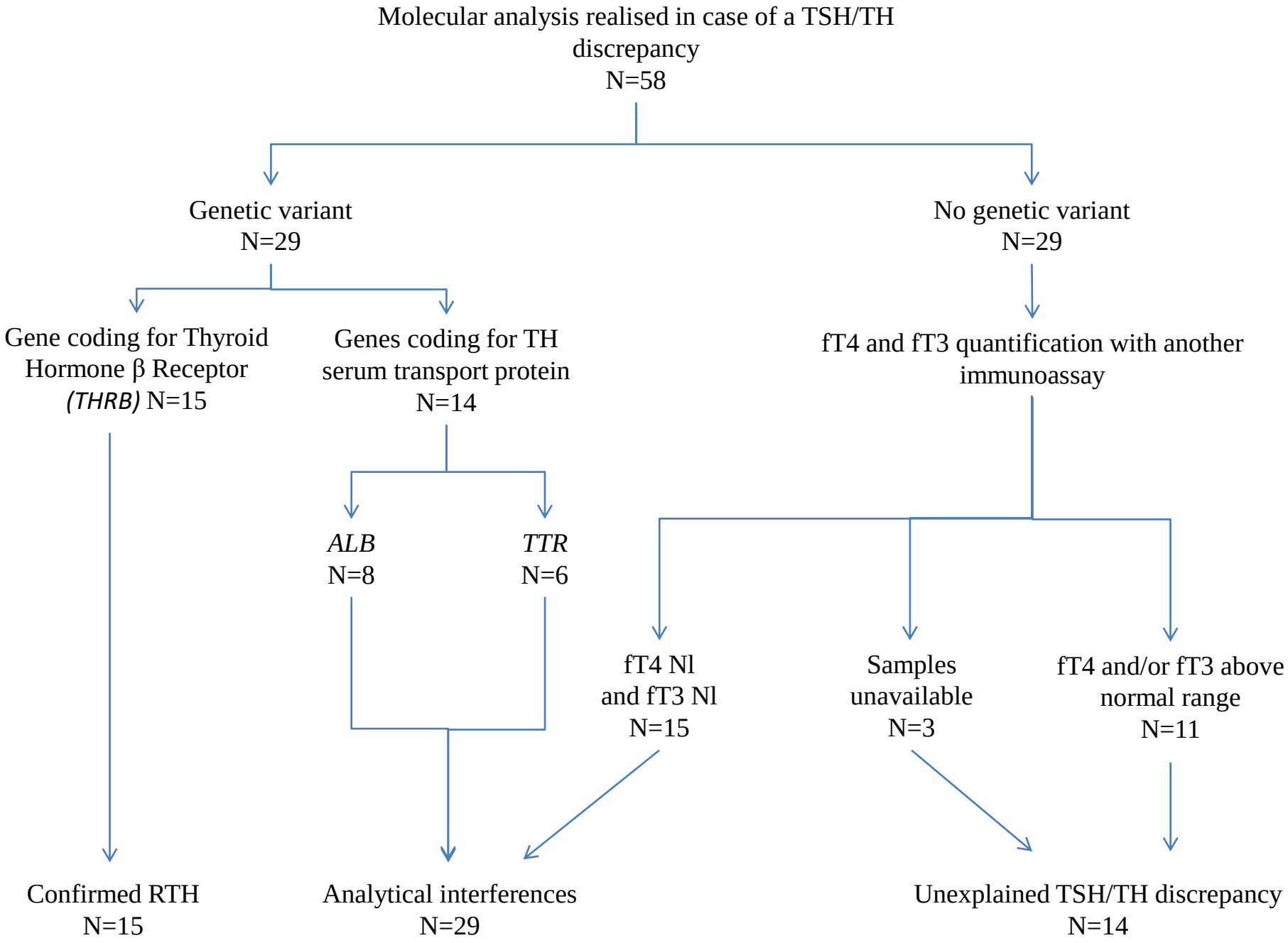
Figure 2 : Boxplots representing age at diagnosis according to sex and etiology of TSH/TH discrepancy. Points represents each patients age.

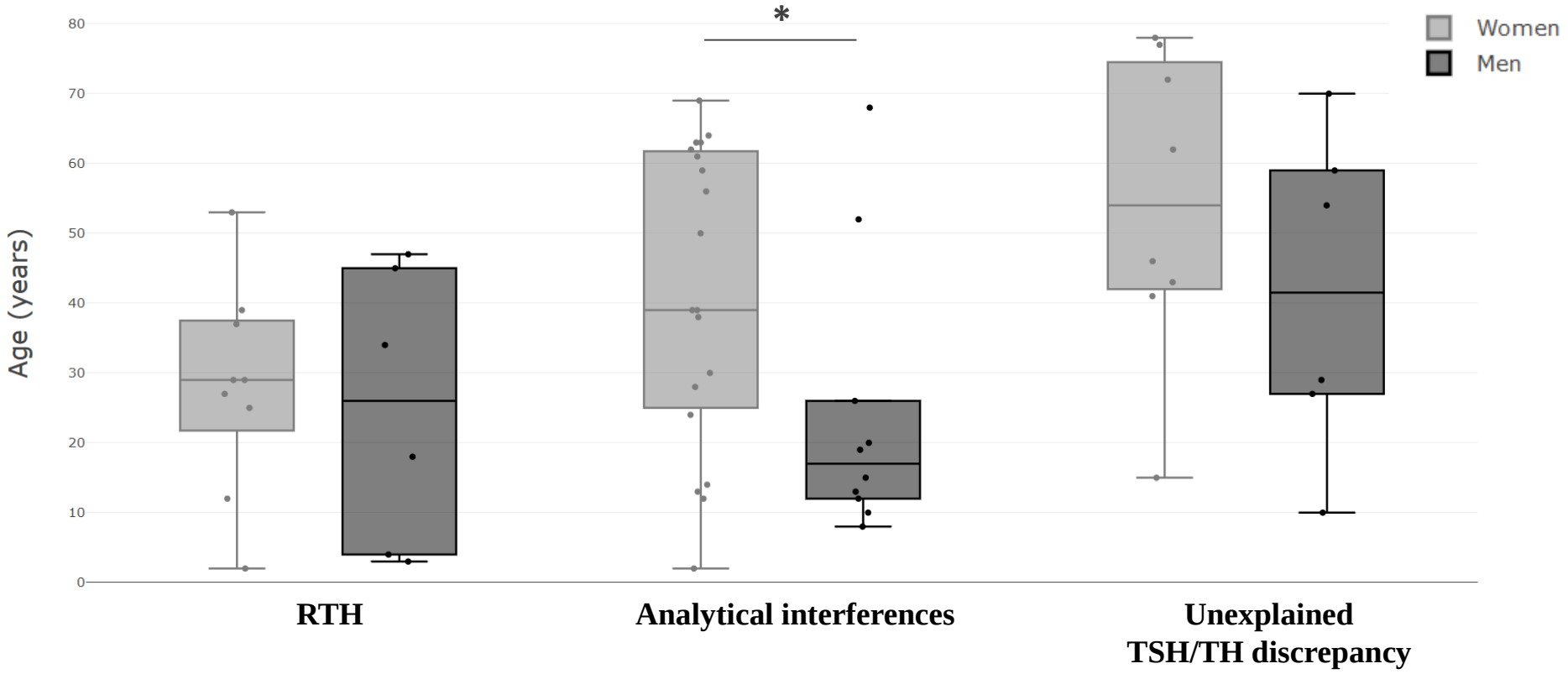
* : p-value = 0.050 (Fisher Exact Test, patients below/above 30 years old according to sex)

RTH: Resistance to thyroid hormones due to a variant of the *THRB* gene, TH: thyroid hormones

Figure 3 : Detail of the different clinical symptoms (A) and distribution of main clinical symptoms (%) (B) according to the etiology of TSH/TH discrepancy.

RTH: Resistance to thyroid hormones due to a variant of the *THRB* gene, TH: thyroid hormones


Molecular analysis realised in case of a TSH/TH discrepancy
N=58
Genetic variant
N=29
No genetic variant
N=29
Gene coding for Thyroid Hormone β Receptor (THRB) N=15
Genes coding for TH serum transport protein
N=14
fT4 and fT3 quantification with another immunoassay
ALB
N=8
TTR
N=6
fT4 Nl and fT3 Nl
N=15
Samples unavailable
N=3
fT4 and/or fT3 above normal range
N=11
Confirmed RTH
N=15
Analytical interferences
N=29
Unexplained TSH/TH discrepancy
N=14


*
Women
Men
Age (years)
80
70
60
50
40
30
20
10
0
RTH
Analytical interferences
Unexplained
TSH/TH discrepancy

clinical symptoms according to the etiology of TSH/TH discrepancy		
Clinical symptoms (number of cases)		**Number of cases**
Confirmed RTH (N=15)		
Goiter	goiter (1), multinodular goiter (1); goiter with nodules (2)	4
Cardiac symptoms	Tachycardia (2), atrial fibrilation (1)	3
Hyperactivity		1
fortuitous discovery		2
Not done		5
Analytical interferences (N=29)		
Goiter	goiter (3),goiter with nodules (1)	4
Cardiac symptoms	tachycardia (9)	9
Hyperactivity		4
fortuitous discovery		13
Not done		3
Unexplained TSH/TH discrepancy (N=14)		
Goiter	goiter (2), multinodular goiter (2); goiter with nodules (1)	5
Cardiac symptoms	Tachycardia (3), atrial fibrilation (2)	5
Hyperactivity		3
fortuitous discovery		2
Not done		1

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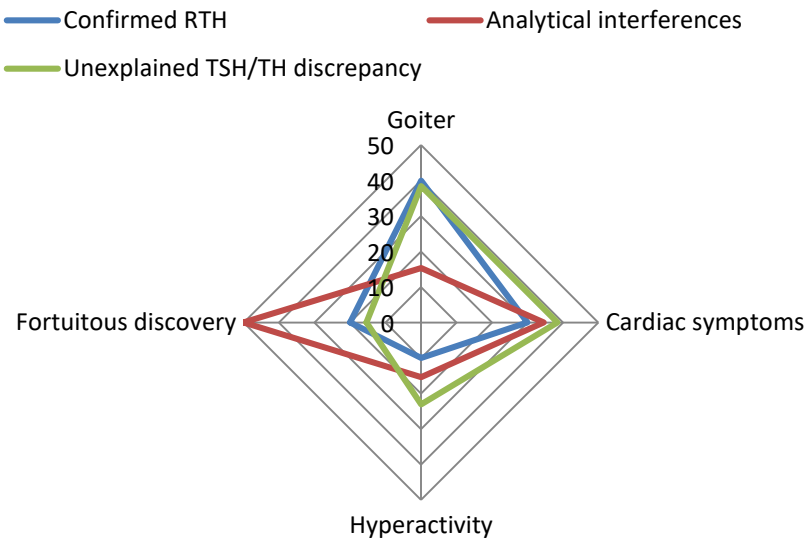

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