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Thyroid Stimulating Hormone Receptor Mutations in Non-Autoimmune Hyperthyroidism

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Thyroid Stimulating Hormone Receptor Mutations in Non-Autoimmune Hyperthyroidism

by

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A THESIS

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Abstract

Non-autoimmune hyperthyroidism (NAH) is rare and occurs due to a constitutively activating thyroid stimulating hormone receptor (TSHR) germline mutation. Germline mutations in TSHR lead to sporadic and familial NAH (SNAH, FNAH) whereas somatic mutations lead to hot thyroid adenoma (HTA). The role and prevalence of TSHR mutations in NAH have been reported to vary significantly. Furthermore, the result of these mutations appears to vary across different reports. Most interestingly, there is also a proposed role for TSHR in thyroid carcinoma. This thesis seeks to determine the true prevalence of TSHR mutations in HTA (the subset of NAH where the most samples are available), explore the phenotype of germline NAH, provide an overview of all TSHR associated disorders and begin to unravel the role of TSHR in carcinoma. This is done in 4 chapters. The first uses targeted NGS technology to determine the true prevalence of TSHR mutations in NAH (specifically HTA). This found that TSHR is the sole gene responsible for the development of HTA (96% mutation positive in an optimal subset of samples). The second chapter explores the phenotype of germline NAH, the variability of presentation, the consequences of late diagnosis, and the possible role of TSHR in bone through literature review and two novel case reports. The third chapter is an all-encompassing look at disorders associated with the TSHR including thyroid carcinoma, as documented by the TSHR mutation database. Thyroid carcinoma is further explored in the fourth chapter which outlines preliminary results and background for a plan to further evaluate TSHR's role in thyroid carcinogenesis. This thesis concludes that TSHR signaling is solely responsible for HTA, that NAH can have variable presentations and requires early total thyroidectomy, and that TSHR undeniably plays a role in thyroid carcinoma that warrants further exploration.

Preface

This thesis consists of a published manuscript (1), two case reports submitted for publication, and a published commentary (2).

CHAPTER 1 serves as an introduction to the thyroid stimulating hormone receptor (TSHR) and to non-autoimmune hyperthyroidism.

CHAPTER 2 is published as "Sensitive Sequencing Analysis Suggests Thyrotropin Receptor and Guanine Nucleotide-Binding Protein G Subunit Alpha as Sole Driver Mutations in Hot Thyroid Nodules" in *Thyroid*. Due to copyright, the version included in this thesis is the pre-print (DOI: 10.1089/thy.2019.0648). Modifications to the pre-print version have been made for this thesis.

CHAPTER 3 consists of case reports for NAH patients with TSHR mutations detected by our lab and literature review of other NAH cases. The reports have been submitted to European Thyroid Journal and Hormone and Metabolic Research respectively.

CHAPTER 4 is published as "The Thyrotropin Receptor Mutation Database Update" in *Thyroid*. Due to copyright, the version included in this thesis is the pre-print (DOI: 10.1089/thy.2019.0807). Modifications to the pre-print version have been made for this thesis.

CHAPTER 5 explores preliminary unpublished data and existing literature for thyroid carcinoma and TSHR.

CHAPTER 6 summarizes key findings of the thesis.

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I would like to express my gratitude to my supervisor Dr. Ralf Paschke for the opportunity to pursue this research project. Thank you for always challenging me to do and think more. I also would like to thank Dr. Markus Eszlinger for his ongoing support with my project, for his open-door policy and for being a friendly face in the lab every single day. I'm incredibly grateful to my lab mate, Paul, for his ongoing assistance with everything from PCRs to math and for his great company. I want to thank the undergraduates who worked on my project, especially Zoya who is invaluable as a teammate and a friend and cannot be thanked enough.

I am also extremely grateful to my supports outside the lab. Thank you to my family and friends, especially Cody, Jahanara, Krista and my parents, for your unwavering support and belief in my abilities.

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List of Abbreviations

THR: thyroid hormone receptor

SNAH: Sporadic congenital non autoimmune hyperthyroidism

TSHR: thyroid stimulating hormone receptor

FNAH: familial non autoimmune hyperthyroidism

TSH: thyroid stimulating hormone

cAMP: cyclic adenosine monophosphate

KI: knock in

HTA: hot thyroid adenoma

tNGS: targeted next generation sequencing

HRM PCR: high resolution melting pcr

DGGE: denaturing gradient gel electrophoresis

HTC: hot thyroid carcinoma

T3: triiodothyronine

T4: tetraiodothyronine

MAPK: mitogen-activated protein kinase

GNAS: G_s alpha subunit protein

PTC: papillary thyroid carcinoma

CHAPTER ONE: INTRODUCTION

1.1 Signal Transduction in the Thyroid

Endocrine glands release hormones into the bloodstream in response to specific stimuli. Receptors located on either the cell surface or inside the cell allow signaling transduction to induce the next step, whether that is the release of a further hormone or a physiological change (3). The thyroid is the endocrine gland responsible for the production, storage and release of thyroid hormones: triiodothyronine (T3) and tetraiodothyronine (T4). These regulate growth, energy metabolism, and oxygen consumption. Their regulation is critical as these hormones induce numerous signaling pathways across multiple organs in the body (4). There are two thyroid hormone receptor (THR) genes: THR-β and THR-α, both of which have three different splice products. The patterns of expression and roles of these forms vary (5). Interestingly, while patients with THR-β mutations have increased thyroid hormone production and goiter development, patients with THR-α mutations have disruptions in normal growth and gastrointestinal function without significant effects on the hypothalamic-pituitary-thyroid axis (6).

Growth and function of the thyroid are controlled by thyroid stimulating hormone (TSH)(7). When there are low levels of serum thyroid hormone, TSH is secreted by the anterior pituitary (5). The TSH receptor (TSHR) is a G protein coupled receptor located on the thyroid follicular cell basolateral membrane with strong evidence supporting its signaling through G_s alpha subunit protein (GNAS)(8)(Figure 1). TSH/TSHR generally controls iodine metabolism but only affects growth in the adult thyroid gland and not during embryonic development(9, 10). It is widely accepted that cAMP stimulates proliferation in the thyroid gland (11-14). Although the activation of the TSHR preferentially stimulates the adenylyl cyclase via the GNAS, at higher TSH

concentrations an activation of the phospholipase C cascade by $G_{q/11}\alpha$ has also been shown (Figure 2) (7, 15). Additionally, there is evidence that the TSHR may be coupled to other members of the G protein family(7, 16). The downstream effects of signaling through different pathways has been investigated, although there is much left unknown. Transgenic models have shown that chronic *in vivo* stimulation of the cAMP cascade stimulates epithelial cell proliferation *in vivo* and also leads to hyperthyroidism (17-19). The role of the TSHR activation of the $G_{q/11}$–phospholipase C (PLC), Ca^{2+} diacylglycerol cascade has been investigated less fully (Figure 2). In human thyroid, defects in $G_{q/11}$ stimulation and consequently of iodination, lead to an important compensatory TSH stimulation, but not goitrogenesis (20). Kero et al. have shown that the $G_{q/11}$-mediated signaling pathway plays an essential role in the regulation of thyroid function. Mice lacking the α subunits of G_q and G_{11} specifically in thyroid epithelial cells showed severely reduced iodine organification and thyroid hormone secretion in response to TSH, and many develop hypothyroidism within months after birth (21). In addition, thyrocyte-specific $G_{q/11}\alpha$-deficient mice lacked the normal proliferative thyroid response to TSH or goitrogenic diet, indicating an essential role of this pathway in the adaptive growth of the thyroid gland (21). Both GNAS and $G_q/_{11}\alpha$ signaling are clearly essential for normal thyroid function and can be associated with hyperthyroidism. TSH and TSHR activation lead to thyroid hormone synthesis via upregulation of sodium iodine symporter, thyroid peroxidase, and thyroglobulin, all of which are essential for the formation of thyroid hormones(22).

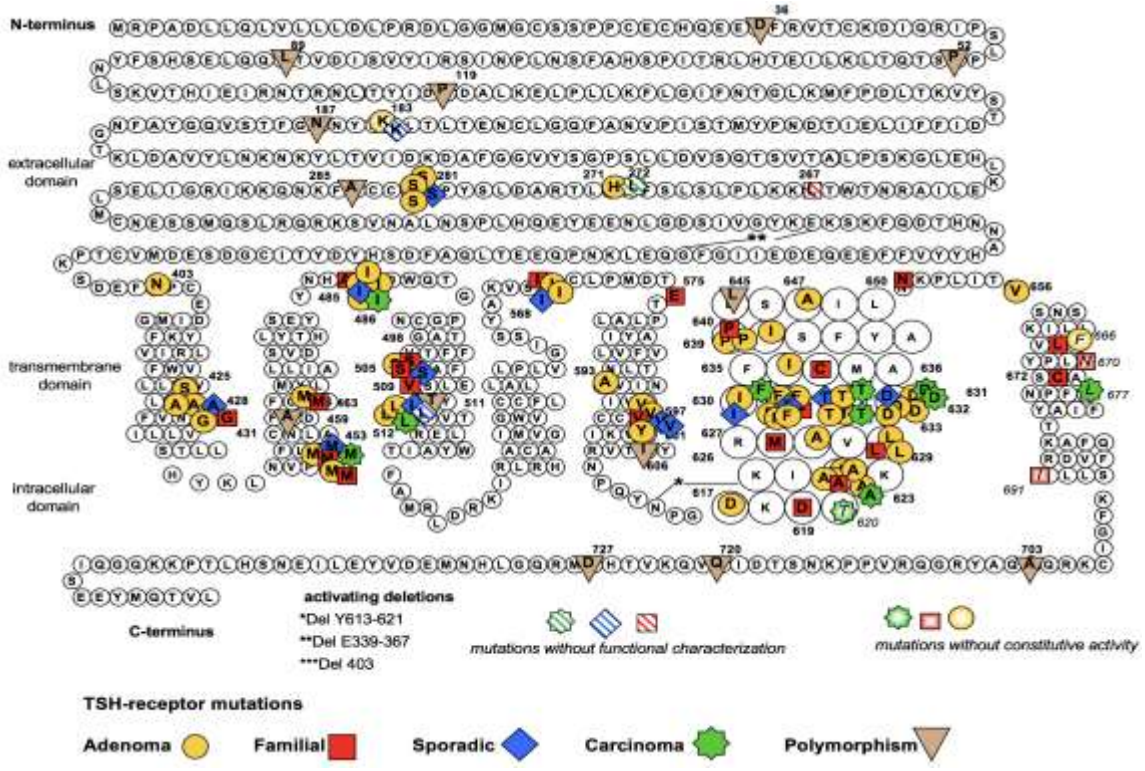

Figure 1: Structure of TSHR with all known activating mutations as documented by the TSHR mutation database.

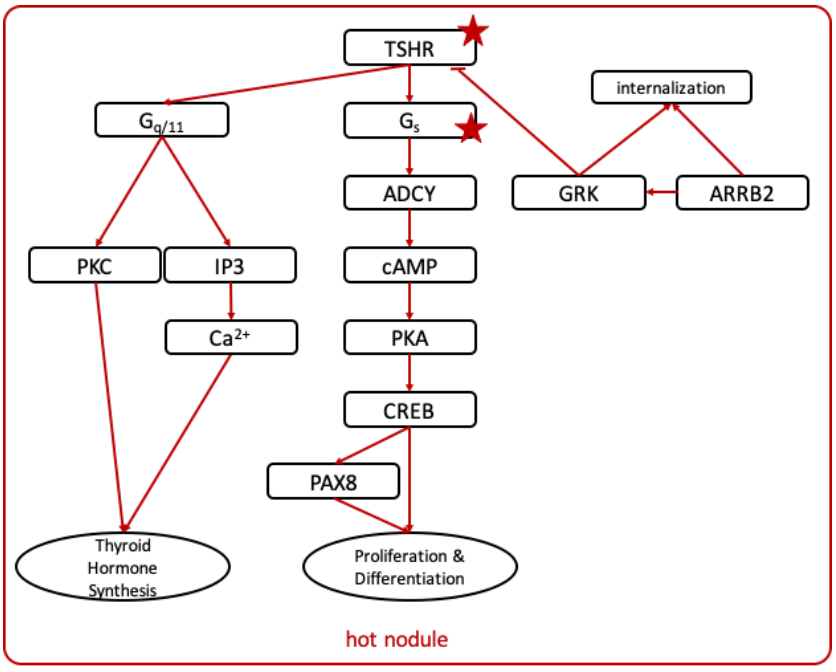

Figure 2: Signaling Cascade for TSHR based on references in introduction. PKC is protein kinase C, IP3 is inositol triphosphate, ADCY is adenylyl cyclase, cAMP is cyclic adenosine monophosphate, PKA is protein kinase A, CREB is cAMP response element-binding protein, PAX8 is paired box 8, GRK is G protein-coupled receptor kinase, ARRB2 is beta-arrestin 2.

The TSHR itself is subject to regulatory mechanisms that could contribute to the etiology and clinical phenotype of TSHR associated disorders. Findings showing an upregulation of beta-arrestin 2 in hyperthyroidism (23) and a predominant desensitization and internalization of the TSHR by beta-arrestin 2 (24) resulted in the assumption that beta-arrestin expression leads to the desensitization of the TSHR and thereby to down regulation of the constitutive activation. However, it has more recently been shown that the interaction of the TSHR with beta-arrestin 2 does not prevent coupling to GNAS and cAMP signaling (25). In addition to their originally described roles in uncoupling G protein coupled receptors from their cognate G proteins and in targeting receptors for endocytosis, beta-arrestins increasingly appear to be directly involved in G protein coupled receptor signaling. Through their ability to recruit SRC kinases, and to act as scaffolds for the ERK and JNK mitogen-activated protein kinase (MAPK) cascades, the binding of beta-arrestins serves to trigger a second wave of signals emanating from the receptor. Thus, beta-arrestins, like heterotrimeric G proteins, link G protein coupled receptors to a defined subset of effector enzymes (26). In addition, a complementary activation of the MAPK pathway by cAMP via EPAC and Rap1 has been proposed in cell lines and in mice (27, 28). Another study showed that only Rap1b is relevant in follicular thyroid carcinoma (29).

Finally, in addition to the intracellular signaling network that is connected to the TSHR, the extracellular action of different growth factors enhances the complexity of the signal flux into the thyroid cell. Growth factors like insulin-like growth factor I (IGF-1), epidermal growth factor, transforming growth factor β, and fibroblast growth factor stimulate growth and dedifferentiation of thyroid epithelial cells(30, 31). Studies, which have been focused on insulin and IGF-1, show a permissive effect of insulin and IGF-I on TSH signaling (32-36) and a cooperative interaction of TSH and insulin/IGF-I (37).

1.2 Non-autoimmune hyperthyroidism

According to the Thyroid Foundation of Canada, thyroid disease is found in 0.8-5% of Canadians and is 4 to 7 times more common in women(38). A major category is hyperthyroidism, defined by the phenotypic excess thyroid hormone production, or in the case of subclinical hyperthyroidism, elevated TSH levels (39). Hyperthyroidism can be classified as autoimmune, or less commonly as non-autoimmune (NAH). Autoimmune hyperthyroidism is known as Graves' disease and involves the production of anti-TSHR antibodies (TRAb), which hyper-stimulate the TSHR leading to increased growth and function. NAH has similar symptoms but without the presence of autoimmine antibodies. There are three categories of interest within NAH : hot thyroid adenoma (HTA), defined by a distinct hyperfunctioning neoplasm; familial NAH (FNAH), defined by autosomal dominant activating germline mutations present in at least two generations; and sporadic congenital NAH (SNAH), defined by sporadic activating mutations present in only the index patient (40).

HTA is named for its visual representation as a "hot spot" on a scintigraphy scan as it takes up an increased amount of radioactive iodine due to its hyperfunctionality. Somatic point mutations that constitutively activate the TSHR were first identified in HTA (41). Subsequently, TSHR germline mutations were identified (42). Somatic and germline TSHR mutations show similar gene expression profiles(43). Current evidence suggests that these mutations explain the clinical phenotype of HTA through their activation of the G_s or the G_s and $G_{q/11}$ pathway, resulting in a stimulation of proliferation and differentiation/hormone synthesis (7, 9, 40, 44). In addition to *TSHR* mutations, mutations in GNAS can also result in an HTA phenotype (41). These mutations are specific to two loci, c. 201 and c. 227. The effect of TSH binding on the structure of the receptor and the specific conformational changes that lead to activation of the G proteins are not known(45).

Treatment for germline and somatic mutations vary. Patients with germline mutations require complete thyroidectomy while somatic mutations resulting in HTA can be treated with removal of the nodule or lobe in which it is found.

1.3 Thyroid Carcinoma and the TSHR

Papillary thyroid carcinoma (PTC) is the most common type of thyroid cancer. Although PTC encompasses several pathological tumor types that have mutually exclusive mutations (ie. Classic PTC, follicular variant PTC), all these mutations signal through the MAPK pathway. The most prevalent mutation is the BRAFV600E mutation (predominantly occurring in classic PTC), which accounts for 61% of mutations, followed by mutations in the NRAS/HRAS/KRAS genes (predominantly occurring in follicular variant PTC), accounting for 13% of mutations (46). Additionally, there are chromosomal rearrangements in about 12% of PTCs resulting in the

illegitimate expression of the kinase domains of BRAF, RET, NTRK or ALK (46). A recent comprehensive genomic characterization of PTC reduced the mutation negative tumors to less than 4% (46). This study also demonstrated striking signaling differences in RAS- and BRAFV600E-driven PTCs: while BRAFV600E-like-PTCs signal preferentially through MAPK, RAS-like-PTCs signal through both MAPK and PI3K. Both mutations induce carcinogenesis through activation of the MAPK pathway, constitutive activation of which is thought to be essential for PTC (47).

TSHR signaling has also been proposed to have a role in MAPK activation. MAPK signaling has been shown downstream of cAMP activation, as activated by TSHR(27, 28). TSHR signaling has even been suggested to have a role in a genetic predisposition to PTC (48). Furthermore, high-normal serum TSH is associated with a higher risk for differentiated thyroid cancer in thyroid nodule patients (49). There are 21 published cases of TSHR mutation positive hot thyroid carcinomas. Furthermore, Franco et al. showed the relevance of Tshr signaling for the development of PTC *in vivo*. While mice with a thyroid-specific knock-in (KI) of oncogenic BrafV600E resulted in invasive carcinomas with a very short latency, the crossing of these mice with Tshr-knock out mice resulted in a partially blocked Braf-induced thyroid growth due to the ablation of Tsh signaling. BrafV600E KI mice were crossed with Gnas-E1fl/fl mice, in which the targeted GNAS allele is inactivated by Cre-mediated recombination. These mice showed characteristics similar to a less risky (1% mortality), low-grade PTCs in humans (less mitosis, no tall-cell features as found in the BrafV600E PTCs)(50, 51) implicating that TSHR signaling may cause a more severe carcinoma phenotype. An additional mouse model showed that increased Tsh concentrations lead to an increased carcinoma prevalence. However, when the Tshr was knocked out, this model no longer developed carcinoma, demonstrating once more the TSHR's relevance in carcinoma

development (52). In summary, these data impressively demonstrate a key role of the Tshr-GNAS-cAMP pathway in Braf-induced PTC initiation, and support the association of TSH levels with thyroid cancer incidence.

1.4 Aims and Hypothesis

This thesis seeks to determine the prevalence and consequences of TSHR mutations in benign non-autoimmune hyperthyroidism, and explore the role of TSHR in thyroid carcinoma. This will be achieved through next generation sequencing of HTA, examining literature for NAH cases and describing two novel cases, updating the TSHR mutation database, and beginning to assess TSHR's role in thyroid carcinoma with patient samples and a mouse model. We hypothesize that THSR signaling alone is responsible for NAH and that TSHR plays an essential role in thyroid carcinoma.

1.5 Statement of contribution

For the "Sensitive Sequencing Analysis Suggests Thyrotropin Receptor and Guanine Nucleotide-Binding Protein G Subunit Alpha as Sole Driver Mutations in Hot Thyroid Nodules" manuscript that constitutes chapter 2, the contributions are as follows: Alexandra Stephenson prepared the manuscript, selected and prepared samples for analysis tNGS and some for HRM. Markus Eszlinger assisted in manuscript preparation, particularly during a lengthy revision process and aided Alexandra in assessing samples. Paul Stewardson designed the tNGS panel with the assistance of Markus Eszlinger and JB McIntyre. JB McIntyre ran the tNGS analysis and assisted

with analysis. Eileen Boesenberg analysed the majority of the samples by HRM and sent samples to Calgary. Rifat Bircan, Seda Sancak and Julya Gozu collected the samples. Sana Ghaznavi and Knut Krohn assisted with editing of the manuscript. Ralf Paschke organized the collaboration, oversaw sample selection and edited the manuscript.

For the "Advanced Bone Age Present in a Neonatal Case of Sporadic Non-Autoimmune Hyperthyroidism Before Onset of Symptoms: A case report" manuscript that constitutes chapter 3, Alexandra Stephenson prepared the manuscript and did a portion of the HRM and Sanger sequencing while training Zoya Punjwani who completed the lab work. Markus Eszlinger oversaw all lab work, verified results and participated in proof reading. Sana Ghaznavi edited the manuscript. Pawel Matusik and Aneta Gawlik are the patient's clinicians. Ralf Paschke oversaw preparation of the manuscript, edited the manuscript and initialized the collaboration.

For the "Report of a further family with two generations with undiagnosed familial non autoimmune hyperthyroidism and review of consequences of late diagnosis of familial nonautoimmune hyperthyroidism" manuscript that constitutes chapter 4, the contributions are as follows: Alexandra Stephenson prepared the manuscript, performed the HRM and Sanger sequencing analysis. Zoya Punjwani extracted the DNA from blood samples. Markus Eszlinger oversaw all lab work, verified results and participated in proof reading. Artur Bossowski is the patients' clinician. Ralf Paschke oversaw preparation of the manuscript, edited the manuscript and initialized the collaboration.

For “The Thyrotropin Receptor Mutation Database Update” manuscript that constitutes chapter 5, the contributions are as follows: Alexandra Stephenson did the literature review, updated the website and wrote the manuscript. Lorraine Lau edited the manuscript. Markus Eszlinger edited the manuscript and assisted with preparation. Ralf Paschke edited the manuscript, assisted with preparation and started the database.

Permission from all co-authors for inclusion of works along with explanation of rules for publications from *Thyroid* in thesis is found in Appendix A.

CHAPTER 2: Sensitive Sequencing Analysis Suggests Thyrotropin Receptor and Guanine Nucleotide-Binding Protein G Subunit Alpha as Sole Driver Mutations in Hot Thyroid Nodules

2.1 Relevance

For decades the etiology of many thyroid disorders has been debated. The molecular etiology of non-autoimmune hyperthyroidism has been investigated with no true conclusion as to whether genes other than TSHR and GNAS are responsible for the phenotype. Finally, in the below paper, we were able to draw the conclusion that non-autoimmune hyperthyroidism is a TSHR mediated disorder with TSHR and GNAS mutations solely responsible for the phenotype.

2.2 Introduction

Constitutively activating mutations in the thyroid stimulating hormone receptor (*TSHR*) are the primary cause of hot thyroid adenoma (HTA) (53, 54). TSHR mutations explain the clinical phenotype of HTA through their activation of the G_s or the G_s and $G_{q/11}$ pathways, resulting in a stimulation of proliferation, differentiation, and thyroid hormone synthesis (21). Sanger sequencing was the original method of detection used for TSHR mutations(55). Due to the low sensitivity and the variable extent of sequencing (e.g., only parts of exon 10 versus exons 9 and 10

sequenced), variable frequencies for *TSHR* mutations in HTA have been previously described. The prevalence of *TSHR* mutations in HTA has been reported to vary from 8 to 82% (41, 55-66). In order to increase sensitivity of detection, ease of application, and cost effectiveness, denaturing gradient gel electrophoresis (DGGE) was employed (67). Despite the increased sensitivity of DGGE (67), a comprehensive study revealed a frequency of only 57% *TSHR* mutations in 75 consecutive HTA (68). Further increased sensitivity and higher throughput was achieved by the use of high resolution melting (HRM) PCR. In addition to higher sensitivity, the advent of HRM-PCR offered further improvements of detection of somatic mutations through simplification of analysis. The use of HRM-PCR showed higher frequency of *TSHR* mutations of 66% in 33 HTA (69).

In addition to *TSHR* mutations, mutations in the G_s alpha subunit protein (*GNAS*) can also result in an HTA phenotype (41). The prevalence of *GNAS* mutations in HTA has been reported to vary from 8 to 75% (41, 55-66). These mutations are specific to two hotspot loci, c. 201 and c. 227. Sanger sequencing or pyrosequencing have been the primary methods of detection of *GNAS* mutations in HTA.

Much of the reported variability in the prevalence of *TSHR* and *GNAS* mutations in HTA can likely be attributed to the sensitivity of the detection method used. In order to determine whether another gene may be responsible for the remaining *TSHR* and *GNAS* mutation negative cases of HTA, whole exome sequencing (WES) was performed for 13 *TSHR* and *GNAS* mutation negative samples (70). This study found no further causative mutation for HTA. Therefore, by using the more sensitive and comprehensive targeted next generation sequencing (tNGS), our aim was to

determine an accurate prevalence of *TSHR* and *GNAS* mutations based on increased sensitivity and coverage. This would allow us to further evaluate the impact of these mutations on the molecular etiology of HTA in a large cohort of HTA derived from two different regions in Europe (Turkey and Germany) (68, 71, 72).

2.3 Materials and Methods

2.3.1 DNA Samples

In the present study, DNA from fresh frozen tissue samples of 148 HTA from three studies performed by Trulzsch et al., Gozu et al., and Sancak et al. were re-analyzed (68, 71, 72). At the time of the first analyses (68, 71, 72), fresh frozen tissue samples derived from the HTA were grinded, homogenized and DNA was extracted from a portion of the homogenized tissue. If DNA from these initial studies was no longer available for the present investigations, DNA was re-extracted from the original homogenized tissue samples. The samples analyzed were 56 samples from Germany (Trulzsch et al.) and 92 samples from Turkey (60 samples from Gozu et al. and 32 samples from Sancak et al.). Samples excluded were 12 *TSHR* and *GNAS* mutation negative HTA from these studies for which no adequate DNA or frozen tissue were available, and 7 mutation positive HTA from the previous publications which could not be matched to the tissues. An additional 34 frozen samples were collected after publication of these studies by the investigators. This included 14 samples from Germany and 20 samples from Turkey (11 from Sancak et al. and 9 from Gozu et al.). Genomic DNA was extracted from the additional frozen tissue samples after grinding and homogenizing the tissue samples using the QiaAmp DNA kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Tissue samples collected post publication had undergone only HRM-PCR analysis prior to tNGS. The additional DNA samples were also

included for a total of 182 HTA analyzed to calculate prevalence of *TSHR* and *GNAS* mutations in HTA. All samples from the study by Trulzsch et al. (68) were from solitary autonomous nodules. Fifteen samples from Sancak et al. (72) were obtained from solitary autonomous nodules, and the additional 17 samples were from toxic multinodular goiter (TMG). Fifteen samples from Gozu et al. (71) were obtained from solitary autonomous nodules and the additional 45 samples were obtained from TMG. The study was approved by the local ethics committees. Written informed consent was obtained from all patients before study participation.

2.3.2 Denaturing gradient gel electrophoresis (DGGE)

PCR, DGGE, and sequencing analyses were performed as described previously by Trulzsch et al. (68). PCR products showing mutations in exons 9 and 10 of the *TSHR* gene by DGGE were subsequently sequenced using Big Dye-terminator chemistry (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions and analyzed on an automatic sequencer ABI 3130xl (Applied Biosystems, Darmstadt, Germany).

2.3.3 High Resolution Melting PCR

Previously DGGE mutation negative samples were re-evaluated using the more sensitive HRM-PCR. *TSHR* point mutations were detected by real time PCR and HRM, using primers reported in the study by Eszlinger et al. (69), encompassing exons 9 and 10 (those exons in which all the previously reported constitutively activating *TSHR* mutations were detected) using the LightCycler 480 High Resolution Melting Master chemistry (Roche, Mannheim, Germany) on a LightCycler 480 (Roche, Mannheim, Germany). PCRs to detect *TSHR* point mutations were processed through an initial denaturation at 95 °C for 10 min followed by 55 cycles of a 3-step PCR, including 3 s of

denaturation at 95°C, a 10 s annealing phase at 56°C, and a 10 s elongation phase at 72 °C. Thereafter, a high resolution melting curve was assessed from 75°C to 95°C with an increase of 0.02°C/s and 25 acquisitions per degree.

2.3.4 Sanger Sequencing

DNA from samples that tested positive by HRM-PCR for *TSHR* mutations were subsequently sequenced using Big Dye-terminator chemistry (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions and analyzed on an automatic sequencer ABI 3730xl (Applied Biosystems, Darmstadt, Germany). The same methodology was applied for the known hotspots c.201 and c.227 of *GNAS*.

2.3.5 Pyrosequencing

GNAS point mutations in position c.201 and c.227 were detected by pyrosequencing using self-designed primers and the following PCR conditions: PCRs were processed through an initial denaturation at 95°C for 15 min followed by 42 cycles of a 3-step PCR, including 20 s of denaturation at 95°C, a 30 s annealing phase at 60°C, and a 30 s elongation phase at 72°C followed by a final 5 min extension phase at 72°C. PCR products were immobilized to streptavidin sepharose beads and single stranded DNA was prepared allowing subsequent annealing of the sequencing primer to the template DNA. Then, the primed single stranded DNA was released from the streptavidin surface and transferred to a PyroMark Q24 (QIAGEN, Hilden, Germany) for pyrosequencing (69).

2.3.6 Targeted Next Generation Sequencing

A custom Ampliseq panel (ThermoFisher Scientific Inc, Waltham, MA, USA) was designed using AmpliSeq Designer software to detect variants in the entire *TSHR* coding sequence and exons 8 and 9 of *GNAS*. Panel size was 4.62 kb with 24 amplicons divided amongst two pools. For each pool, sample libraries were generated from 10 ng of DNA using Ion AmpliSeq Library Kit 2.0 (ThermoFisher Scientific, Waltham, MA, USA) and barcoded with Ion Xpress barcode adapters (ThermoFisher Scientific, Waltham, MA, USA). Sample pools were combined and quantified using the Agilent High Sensitivity DNA kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Sample libraries were then combined and diluted to 30pM and loaded on the Ion Chef instrument for automated template preparation and chip loading onto an Ion 520 chip. Loaded chips were sequenced using 200bp sequencing chemistry with 500 flows on an Ion S5 XL sequencer (ThermoFisher Scientific, Waltham, MA, USA). Sequencing reads were generated with Torrent Suite software v5.8 (ThermoFisher Scientific, Waltham, MA, USA) and aligned to human reference genome 19 (hg19). Variant calling was performed with the Torrent Variant Caller plugin and annotated using Ion Reporter v5.10. Briefly, variants were called using somatic stringency parameters optimized for low frequency variant detection >2.5%. All variants were manually reviewed using Integrated Genomics Viewer (73). Mean depth of sequence was ~8,800x.

2.3.7 Relevance of novel TSHR mutations

Novel TSHR mutations were evaluated based on SIFT and PolyPhen2 scores to predict whether amino acid substitutions were deleterious (74, 75).

2.3.8 Statistical analysis

Pearson's Chi-squared test and Fisher's Exact Test were used as appropriate to determine difference in frequencies using standard R packages.

2.4 Results

2.4.1 Prevalence of TSHR and GNAS Mutations in HTA

The aim of this study was to accurately determine the true prevalence of *TSHR* and *GNAS* mutations in HTA. To do so, we analyzed 182 HTA by combining the previously published DGGE and Sanger sequencing results (68, 71, 72) with results obtained by DGGE in 34 samples collected following our publication, HRM-PCR and pyrosequencing of 23 DGGE mutation negative samples collected following our publication and of 67 published DGGE mutation negative samples, and tNGS for 38 HRM-PCR and pyrosequencing mutation negative samples, 4 of which were collected after publication (68, 71, 72). Only samples which were mutation negative were included for mutation testing in the next methodology. Samples with positive results were not reanalyzed. Ten samples with known polymorphisms were included in the mutation negative category and reanalyzed.

Of the 67 published samples with wildtype DGGE results, reanalysis showed 20 had *TSHR* mutations detected by HRM-PCR, two had *GNAS* mutations detected by pyrosequencing (completed in Germany by co-authors), six and three samples had *TSHR* or *GNAS* mutations, and one sample had both a *TSHR* and *GNAS* mutation detected by tNGS. Of 34 additional samples collected following our prior publication, 11 samples had *TSHR* mutations detected by DGGE,

four had *TSHR* mutations detected by HRM-PCR, one had a *GNAS* mutation detected by pyrosequencing (this data was collected by co-authors in Germany). No additional *TSHR* mutation was detected by tNGS.

38 HTA were analyzed by tNGS; 34 of these samples were from prior publications and tested as mutation negative by HRM-PCR, while four were collected after our publication (68, 71, 72). The 34 previously published HTA were earlier found to be *TSHR* and *GNAS* mutation negative as tested by Sanger sequencing, DGGE, HRM-PCR and pyrosequencing. Of these 34 HTA, *TSHR* or *GNAS* mutations could be identified in an additional six and three HTA, respectively, with an additional sample found to have co-occurring *TSHR* and *GNAS* mutations by tNGS (Table 1). Variant allele frequencies of detected mutations varied from 3-37%. Twelve *TSHR* and *GNAS* mutation negative HTA analyzed in the three previous studies did not have remaining DNA for further analysis by tNGS. Therefore, in total 154 HTA were positive for *TSHR* or *GNAS* mutations resulting in an 85% prevalence of *TSHR* and *GNAS* mutations in HTA. In detail, 144 HTA (79% of HTA, 93.5% of mutation positive HTA) were found to have *TSHR* mutations, and 10 HTA (6% of total HTA or 6.5% of mutation positive HTA) were found to have *GNAS* mutations. The number of mutation positive samples was not significantly different between solitary nodules and nodules from TMG (Fischer Exact Test, p=1).

TABLE 1. SOMATIC MUTATIONS IDENTIFIED USING tNGS, ORGANIZED BY ASCENDING VARIANT ALLELE FREQUENCY OF *GNAS* AND *TSHR* MUTATIONS

Sample ID	*DGGE result*	*HRM result*	*tNGS result*	*Variant allele frequency as detected by tNGS, %*	*SIFT/PolyPhen2 scores for newly identified mutations*	*Amino acid change*	*Described in TSHR mutation database*
S15	WT	WT	GNAS c.681G>T	37		GNAS Q277H	n/a
T131	WT	WT	GNAS c.681G>T	25		GNAS Q227H	n/a
T136	WT	WT	GNAS c.601C>T	14		GNAS R201C	n/a
S25	WT	WT	TSHR c.1802A>T	35	0.0/1.0	TSHR Y601P	Newly identified
			TSHR c.1458C>G	26		TSHR I486M	Yes
			TSHR c.1358T>C	13		TSHR M453T	Yes
			TSHR c.1535T>A	8		TSHR L512Q	Yes
			TSHR c.1714_1715delATinsGC	6	0.0/0.998	TSHR M572A	Newly identified
			TSHR c.2009A>G	5		TSHR N670S	Yes
S7	WT	WT	TSHR c.1919T>C	5	0.0/1.0	TSHR I640T	Newly identified
G8.2	WT	WT	TSHR c.1658C>T	4	0.0/1.0	TSHR A553V	Newly identified
			TSHR c.1714A>G	4	0.0/0.993	TSHR M572V	Newly identified
S24	WT	WT	TSHR c.1878G>C	4		TSHR M626I	Yes
T73	WT	WT	TSHR c.1387A>G	3		TSHR M463V	Yes
S10	WT	WT	TSHR c.1919T>C	3	0.0/1.0	TSHR I640T	Newly identified
G48.2	WT	WT	GNAS c.602G>A	34		GNAS R201H	n/a
			TSHR c.1919T>C	4	0.0/1.0	TSHR I640T	Newly identified

Samples were separated into three groups: samples with *GNAS* mutations, samples with *TSHR* mutations, and a single sample with both a *TSHR* and a *GNAS* mutation. The first letter in sample ID indicates which publication the sample originated from: S [Sancak *et al.* (22)], T [Trulzsch *et al.* (18)], and G [Gozu *et al.* (21)]. TSHR Mutation database found at tsh-receptor-mutation-database.org, updated 2019. Mutations described in the mutation database have been characterized as constitutively activating. Mutations detected for the first time in this study have SIFT/PolyPhen2 score indicating their relevance (23,24).

DGGE, denaturing gradient gel electrophoresis; GNAS, guanine nucleotide-binding protein G subunit alpha; HRM, high-resolution melting; tNGS, targeted next-generation sequencing; TSHR, thyrotropin receptor; WT, wild type.

2.4.2 Analysis of multiple samples per nodule and intra-nodule mutation variability

For 51 HTA from the study by Gozu and colleagues (71) a single tissue sample per nodule was available for mutation screening. A *TSHR* mutation was detected in 42 of the 51 HTA (82%) and a *GNAS* mutation was detected in one HTA (2%) resulting in a total of 84% mutation positive HTA.

For 25 HTA from the study by Gozu et al. (71) multiple samples from different regions of the same HTA were available for molecular testing. While 18 HTA of this subgroup yielded consistent results for all samples analyzed per nodule, in seven samples discrepant results were detected (Table 2). Of the seven HTA with discrepant results for samples derived from different regions of a nodule, all had at least one sample with a wildtype result (Table 2). While in four cases (G31.2,

G15.3, G4.2 and G24.3) the wildtype mutation status was associated with cystic, necrotic or degenerative features reported by the pathologist during the gross examination of the surgical specimens, the reported cystic degenerative areas reported for nodule G12 cannot precisely be linked to the two wildtype samples G12.2 and G12.3 (Table 2). Interestingly, one 2x2 cm HTA with three available samples from different regions of the nodule was found to have two different *TSHR* mutations as well as one wildtype result (sample G15; Table 2). Overall, a *TSHR* mutation could be detected in 23 of the 25 HTA (92%) and a *GNAS* mutation was detected in 1 HTA (4%) resulting in a total of 96% mutation positive HTA (Figure 3).

TABLE 2. RESULTS OF ANALYSIS BY tNGS AND PATHOLOGY FINDINGS FOR HOT THYROID NODULES FROM GOZU *ET AL.* (21) SAMPLES

Sample ID	*Nodule size*	*Region of the nodule where the tissue sample was taken from*	*Pathology notes*	*Mutations detected*
G7.2	2.5×1.6 cm	Lateral part	No notes for cystic changes	WT
G7.3		Medial part		TSHR N372T
G48.2	3×3 cm	Lateral part	No notes for cystic changes	TSHR I640T GNAS R201H
G48.3		Central part		WT
G31.1	3.5×3 cm	Superior part		TSHR T632A
G31.2		Central part	Cystic degeneration	WT
G12.1	4×4 cm	Superior part	Cystic degeneration	TSHR M453T
G12.2		Lateral part		WT
G12.3		Medial part		WT
G15.1	2×2 cm	Superior part		TSHR S505N
G15.2		Medial part		TSHR V656F
G15.3		Lateral part	Cystic degeneration	WT
G4.1	5×3.5 cm	Central part		TSHR F631I
G4.2		Superior part	Necrosis	WT
G4.3		Inferior part		TSHR F631I
G24.1	6×5.5 cm	Superior part		TSHR T632I
G24.2		Lateral part		TSHR T632I
G24.3		Medial part	Cystic degeneration	WT
G24.4		Inferior part		TSHR T632I

Discrepant results were found when more than one sample was analyzed per hot thyroid nodule.

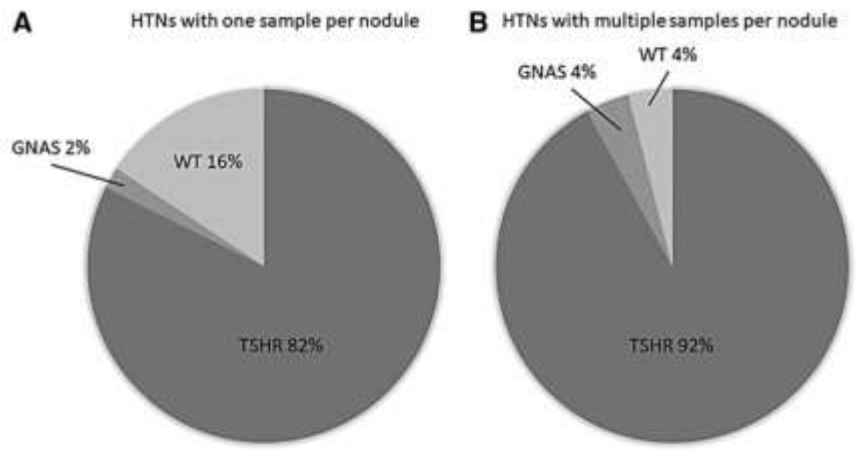

Figure 3. Prevalence of TSHR and GNAS mutations in HTNs from the Gozu et al. sample set. A) HTNs with one sample (n=51) vs. B) HTNs with more than one sample per nodule.

2.4.3 Multiple TSHR mutations in a single nodule detected by tNGS

In addition to HTA G15 with three available samples (G15.1, G15.2, G15.3) from different regions of the nodule (Table 2) two HTA (G8.2 and S25) were found to have more than one *TSHR* mutation in a single sample (Table 1). A single sample from the 2x2cm HTA G8.2 was found to have two *TSHR* mutations by tNGS (Table 1). In addition to these two somatic *TSHR* mutations, tNGS also found a *TSHR* c.154C>A (p.P52T) mutation, which is a known germline polymorphism (76).

Only one tissue sample was retrieved from HTA S25, a 4.3x2.1cm nodule. tNGS analysis of this sample found 6 *TSHR* mutations of various variant allele frequencies ranging from 5 to 35% (Table 1).

Two samples were taken from the 2.8x1.4cm HTA G48. While sample G48.2 taken from the lateral part of the HTA was found to have both a *TSHR* and *GNAS* mutation (Table 1), sample G48.3 taken from the central part of the HTA was wildtype.

2.5 Discussion

2.5.1 Summary

With the aim of determining the accurate prevalence of *TSHR* and *GNAS* mutations and to further evaluate the impact of these mutations on the molecular etiology of HTA, we re-analyzed 38 *TSHR* and *GNAS* mutation negative HTA from 182 HTA previously analyzed by DGGE and HRM using a comprehensive tNGS panel covering the entire coding sequence of the *TSHR* gene and hot spots for *GNAS* constitutively activating mutations. This included 34 previously published *TSHR* and *GNAS* mutation negative HTA and an additional 4 mutation negative HTA collected after the three original publications (68, 71, 72). Overall, our findings show an 85% prevalence of *TSHR* and *GNAS* mutations in HTA. This is significantly higher compared to previous reports: Trulzsch et al. (68), Sancak et al. (72) and Gozu et al. (71) which reported 60%, 43%, and 72% of samples to harbor *TSHR* or *GNAS* mutations, respectively (p-value= 1.187×10^{-7}, Pearson's Chi-squared test). In all subsets, the predominant reason for this increased prevalence is the progressive increase in sensitivity in mutation detection methodologies from Sanger sequencing to tNGS.

2.5.2 Low Variant Allele Frequencies

Based on a mean depth of coverage of ~8,800x in our tNGS panel, it is highly likely that we can detect mutations at frequencies down to three percent. Detection of lower mutation frequencies might have been hampered by PCR error (which is around one percent) due to the fact that we did

not use molecular identifiers to control for this source of error. In comparison to the previously used methods, this increased tNGS sensitivity allows for more accurate detection of mutations in HTA than previously described. Therefore, although we identified low VAFs between 3 and 8% for 11 out of 20 mutations detected by tNGS (Table 1), we do interpret these results as accurate based on our high sensitivity and depth of coverage. Moreover, all of these mutations have been validated through visual inspection of sequencing reads in the Integrated Genomics Viewer (77). The low VAF findings and also wildtype findings could be explained by sampling in an area of the nodule with a low number of mutated cells due to bleeding, necrosis, cystic changes, calcification, degeneration, or other unknown factors contributing to an allele frequency that is not representative of the whole nodule. Furthermore, DNA was extracted from homogenized grinded tissue, therefore low VAFs may be explained by dilution if the mutation was only present in a subclone within the nodule. Available pathology reports of tNGS wildtype samples frequently described at least one of the above characteristics (Table 2). While less likely, contamination by normal thyroid tissue could also lead to decreased VAFs.

2.5.3 Multiple samples per nodule and intra-nodule mutation variability

Of the Gozu sample set (71) seven of 25 HTA with multiple tissue samples per nodule had discrepant results where at least one sample was wildtype (Table 2). Had these wildtype samples been the only samples analyzed, the mutations reported by DGGE, HRM and our tNGS analysis would have been lower in number. The comparison of the subgroups of HTA with one sample (n=51) versus HTA with more than one sample per nodule (n=25) within the Gozu sample set reveals a substantially, but not significant, higher mutation prevalence of 96% versus 84% in the HTA with multiple samples per nodule (Figure 3; p-value=0.26, Fisher's Exact Test for Count

Data). While the analysis of multiple samples per nodule in this sample set explains the greater number of mutations detected, it also substantiates our hypothesis that issues of sampling may lead to undetectable mutation levels in one sample/area of an HTA, and a detectable mutation in another sample/area of the same nodule.

It is important to note that we detected new mutations that have not yet been characterized in cell culture and included those in our prevalence calculations. Based on SIFT/PolyPhen2 scores we can assume that these mutations have an effect on the protein function however without cell culture experiments we cannot make a definitive claim that these mutations are responsible for the hot phenotype. While we are aware of this limitation and its effect on our ability to draw strong conclusions, functional characterization was not the focus of this study and is outside the scope of our aims.

Recently, whole exome sequencing of 13 *TSHR* and *GNAS* wildtype HTA detected no further causative driver mutations which could explain the clinical phenotype of the HTA (70). Although, based on our current findings, the analysis of single samples per nodule seems to be a limitation of that study, the fact that no further driver mutation could be detected by WES supports the hypothesis that *TSHR* and *GNAS* mutations are the only driver mutations in HTA. This hypothesis gains further support by our current finding of a 96% *TSHR*/*GNAS* mutation prevalence in HTA detected in an optimal sample set with multiple samples per nodule and analyzed by a comprehensive tNGS panel with high sequencing depth.

2.5.4 Multiple TSHR mutations in a single nodule detected by tNGS

Of particular interest are the three HTA with more than one mutation. To the best of our knowledge, HTA with multiple *TSHR* mutations in the same nodule have not previously been described. Both mutations (A553V and M572V) detected in HTA G8.2 are newly identified with very low VAFs of 4%. Histology of this HTA described signs of bleeding and cystic areas. Both A553V and M572V have not yet been characterized as constitutively activating and these amino acids have previously not been identified with constitutively activating mutations but are found in exon 10. Based on their SIFT and PolyPhen2 scores both mutations are described with a high probability as deleterious mutations. Interestingly, in amino acid position 553, an inactivating mutation has been previously described four times (78-81). There is one other instance (amino acid 593) in the *TSHR* mutation database (tsh-mutation-database.org) (44) where different mutations at a particular locus are both activating and inactivating. Based on the low variant allele frequency of 4%, it is unclear what the biological relevance of these mutations is. More importantly, the VAF may be heterogeneous throughout HTA and this low VAF finding is not necessarily representative of the entire HTA.

HTA G48.2, was found to have both a previously undescribed *TSHR* I640T (c.1919T>C, VAF 4%) and a *GNAS* R201H (VAF 34%) mutation. While the *TSHR* mutation, I640T, is not found in the *TSHR* mutation database (tsh-mutation-database.org) (44), another somatic mutation, I640K, at this position has been found to be constitutively activating (71). The *GNAS* mutation is known to be constitutively activating. The biologic and signaling effect and the relevance of these co-occurring mutations is not known.

tNGS analysis of HTA S25, a 43x21 mm HTA, found four known constitutively activating *TSHR* mutations with varying VAFs (5-26%), and two additional newly identified mutations TSHR c.1802A>T (Y601P) and TSHR c.1714_1715delATinsGC (M572A) with VAFs of 35 and 6%, respectively (Table 1). For the amino acid position of one of these new mutations (Y601P) a constitutively activating mutation (Y601N) has been described (82). Based on their SIFT (75) and PolyPhen2 (74) scores both mutations are described with a high probability as deleterious mutations. None of these mutations were detected in other samples making contamination error an unlikely reason for this finding. However, the biological relevance, the signaling effect, and the etiologic mechanism of more than one *TSHR* mutation in the same HTA is unknown. The phenotype of this HTA is not different from other nodules in size or hormone levels before treatment onset. The sequencing results alone do not indicate whether the *TSHR* mutations accumulated over time in a cell population derive from a single progenitor or whether the *TSHR* mutations occur in isolated distinct populations although the occurance of different mutations in multinodular specimens may suggest the latter..

2.5.5 Conclusion

In conclusion, due to the higher sensitivity of the tNGS panel compared to DGGE and HRM-PCR, *TSHR* or *GNAS* mutations could be detected in 85% of HTA. In an optimal sample set with multiple samples per nodule analyzed by a comprehensive tNGS panel with high sequencing depth, the prevalence of *TSHR* and *GNAS* mutations rose to 96%. These results, in addition to the fact that no further causative driver mutations explaining the phenotype of HTA could be identified by previous WES, strongly support the hypothesis that *TSHR* and *GNAS* mutations are the only somatic mutations leading to HTA.

CHAPTER 3: CASE REPORTS OF NAH

3.1 Relevance

The conclusion that HTA, a form of NAH, is a TSHR mediated disorder gives strength to our current methodologies for molecular diagnosis for germline NAH. Currently, the absence of TRAb in a patient with hyperthyroidism warrants molecular analysis by HRM PCR and Sanger sequencing. While our study using tNGS demonstrates the superiority of an NGS technology, cost and the timeline-associated limitations due to batch size must also be taken into account. While HRM PCR and Sanger sequencing can be done immediately upon receiving samples, tNGS would require labs to wait until there were multiple patient samples awaiting analysis in order to try to decrease the cost, which would still be significantly higher than HRM PCR or Sanger sequencing. Furthermore, with germline mutations we expect a VAF of roughly 50%. There were no cases in our tNGS publication of HRM PCR false negative results for a sample with this high of a VAF. For these reasons, our method for identification of TSHR mutations in potential NAH patients remains HRM PCR and Sanger sequencing. The knowledge that TSHR alone is responsible for NAH validates our testing methodology of TSHR screening by HRM PCR and Sanger sequencing followed by GNAS screening by Sanger sequencing.

TSHR mutation screening is not offered by hospital laboratories; for this reason, physicians that suspect NAH must send their samples to a research lab for molecular testing. Below, two cases that were sent to our lab for molecular testing are explored. Both their genetic background and the literature surrounding the cases will be examined.

3.2 Case Reports

3.2.1 Sporadic NonAutoimmune Hyperthyroidism

The patient was a female born in the 37th week of gestation in Katowice, Poland. Birth weight was 2600g and birth length was 50 cm. APGAR score was 8 points at the first minute, and 9 points after five minutes. Screening for hypothyroidism at birth is compulsory in Poland but does not detect hyperthyroidism. The patient's mother was diagnosed with transient gestational hypothyroidism at 19 weeks gestation and treated with levothyroxine during pregnancy. Due to this, the neonate's thyroid function was investigated at 2 and 4 months of age, and hyperthyroidism was diagnosed at 4 months based on suppressed TSH and increased free T_3 and T_4 (fT3 and fT4) (Table 3). While the patient showed suppressed TSH at 2 months of age, the general pediatrician initially believed it to be laboratory bias as the patient did not show any signs of hyperthyroidism, and therefore, a referral to a specialist was not initiated at this time.

Weeks of Age	Weight [kg]	Height [cm]	Head circumference [cm]	Notes
16	5.4	67	41	Admission to clinic
17				BA: 1,5 yrs, USG: low heterogenous echogenicity with higher blood flow; RL - 10x12x29 mm; LL - 8x16x28 mm; thyroid volume 3.38mL (Figure 1)
19	6.62			discharge from the clinic
20	6.9	67.5	41.5	first visit in the out-patient clinic
24	7	68	42	
25	8	70	42	
28	8.6	71	43	
33	9.1	74	45	
36	10.2	75	45.5	
46	10.7	80	46	
52	11.5	80	46.5	BA - 2 yrs 6 months; USG: RL - 14,6 x 13,3 x 34,2mm; LL - 13 x 14 x 31,2mm; thyroid volume 5.90mL
56	12	83	47	
64		84	47	
73	12	85	47	
83	13	87	47.5	
90	13.2	89	47.5	BA - 3 yrs; USG: RL - 12,9 x 16,1 x 36,4 mm; LL - 13,1 x 13,5 x 34,5 mm; thyroid volume 6.54mL
94	13	89	47.5	
98	13.5	90.5	48	
103	14.1	91.5	48.5	
107	14.5	92	49	
111	15.3	94.5	49	BA - 4 yrs 2 months; USG: RL - 14,9 x 17,4 x 38,7 mm; LL - 14,7 x 13,4 x 41,2 mm; thyroid volume 8.69mL

Table 3 Patient measurements, notes on bone age (BA) and thyroid ultrasound measurements (USG) of right lobe (RL) and left lobe (LL).

After the hyperthyroidism diagnosis at 4 months, the patient was hospitalized in order to normalize thyroid hormone levels. Physical examination revealed no dysmorphic features and the resting heart rate was normal (heart rate 140/min; normal range: 100-180(83)). The patient had a body length of 67 cm (> 97 percentile), a body weight of 5400g, and a head circumference of 41cm (50 percentile). Anti-thyroid antibodies were negative both for anti-TPO and anti-TSH receptor. Thyroid ultrasound showed her thyroid to be enlarged, with heterogenous echogenicity, some areas of hypoechogenicity, increased vascular flow, and no presence of nodules. The thyroid volume was 3.38 mL at 17 weeks (Table 3) as compared to the average size of 1.1 mL for European newborns (84). The volume of each lobe was calculated by the formula: V(ml)=0.479*d*w*l(cm). The thyroid volume was the sum of the volumes of both lobes (85). An X-ray of the patient's hand and wrist revealed an advanced bone age of 1.5 years at a chronological age of 4 months, using Greulich and Pyle's bone age assessment. The patient was treated with 5 mg/day of thiamazole and was discharged 3 weeks later with test results showing TSH <0.005 μIU/mL (N: 0.270-4.20 μIU/mL; Elecsys TSH assay by Cobas); fT4 – 2.69 (N: 0.65-2.3 Elecsys fT4 III assay by Cobas); fT3 – 6.74 (N: 2.15-5.83 Elecsys fT3 III assay by Cobas).

After several dose increases with transient periods of normalization of thyroid function, the patient failed to go into remission with antithyroidal medications alone (Figure 4). Bone age was measured again at a chronological age of 1 year; at this time, the patient had a bone age of 2.5 years and exhibited a further increase in thyroid gland volume shown by ultrasound (Table 3). Bone age was measured twice more at 21 months and 25 months and continued to increase disproportionately to chronological age (Table 3). Craniosynostosis was not present, evaluated on clinical grounds only. No clinical or biochemical adverse events were observed during the treatment period.

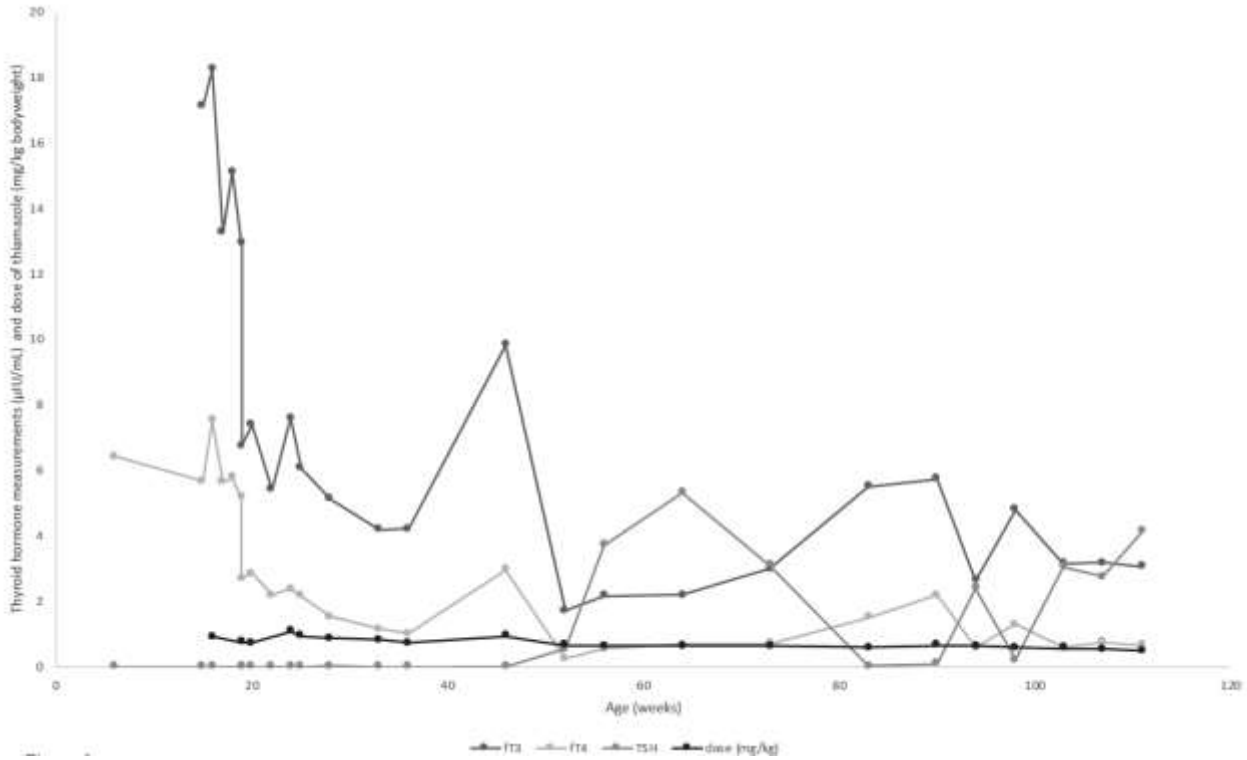

Figure 4 Patient thyroid hormone measurements and thiamazole dose following diagnosis and onset of treatment at 6 and 16 weeks of age respectively.

During the course of her treatment, the patient remained within the normal range for weight, height and head circumference according to the Polish guidelines (86) (Table 3). As the patient's psycho-physical development is normal thus far, the parents and treating physician agreed to delay total thyroidectomy and continue to discuss the patient's parents' concerns regarding anesthesia and post-operative complications. The primary physician continues to monitor the patient's psychological, neurological and anthropometrical state and treat with antithyroid drugs.

3.2.2 Familial NonAutoimmune Hyperthyroidism

The index patient (III/1) is a male, born by Caesarean section at term to unrelated parents. During gestation his mother was treated with antithyroid drugs for hyperthyroidism. Thyroid hormones and antithyroid antibodies were checked because of bradycardia in the newborn and family history of thyroid disorders. At seven days of age, hypothyroidism was diagnosed based on TSH 5.88 μIU/ mL (ref: 0.32-5.0; ECLIA Cobas, Warsaw Poland) At this time, therapy with l-thyroxin (10ug/kg/day orally) was commenced. Following rapid normalization of TSH, normal levels of his antithyroid antibodies (Anti- ATG- 17,29 IU/ml (N: 0- 115); Anti- TPO- 10,04 IU/ml (N: O-34); ECLIA E170, Roche Diagnostics) and normal thyroid gland results by ultrasonography, l-thyroxin dose was decreased until it was discontinued at six months of age. At this time, the patient's motor and mental development was normal. At three years of age, thyroid hormone levels were investigated due to tachycardia and weight loss observed by the mother. Hyperthyroidism was diagnosed based on thyroid hormone results (Table 4). A bone age of seven years was detected based on the Greulich-Pyle method. TPO and ATG were normal (ECLIA E170, Roche Diagnostics). Ultrasound showed normo-echogenic, homogenous thyroid of normal volume (3mL; left lobe: 27x12x10mm; right lobe: 25x11x10mm). The patient was treated with methimazole (1mg/kg/day) and propranolol (1mg/kg/day). After approximately 6 months of therapy the child was hypothyroid (Table 4). The dose of methimazole was reduced and thyroid hormone levels normalized. At four years of age, physical examination found the thyroid to be slightly enlarged (LL 7.2mL; RL 7.0mL (N: <3 whole thyroid (87)). Ultrasound showed enlarged thyroid lobes that were slightly hypoechogenic with a 0.6x0.6cm nodule in the right lobe. At 11 years, 7 months the patient was once again hypothyroid and required a reduction in dose of methimazole (Table 4). Presently, the child is 13 years of age, his height is 90th percentile and his body mass is 50-75th

percentile (86). The most recent thyroid ultrasound measurements at the age of 12 years were RL: 2x2.3x3.55 cm, volume: 8.5 mL; LL- 2x2x4.12cm volume: 8.48 mL (normal lobe volume: 3.3mL; isthmus: 0.34 cm) (88). The thyroid was hypoechogenic at this time. The patient was treated with methimazole (0.1mg/kg/day) and supplemented vitamin B and magnesium while thyroidectomy is planned. While no side effects of antithyroid drug treatment have been observed, significant variations in thyroid hormone levels were an issue throughout treatment.

Age	Weight (kg)	Weight Percentile	Height (cm)	Height Percentile	TSH (μIU/mL)	fT3 (pg/mL)	fT4 (ng/dL)	methimazole dose (mg/kg/day)
2	14.5	90	92	90				
3	20	90-97	107	90-97	0	8.09	2.61	1
3y6m					16.64		0.705	0.5
3y7m					2.03		1.4	
4	22	90-97	112	90	0.005	1.77	5.15	
5	23	90	116	90				
6	26	75-90	126	90				
10y 6m	39	50-75	153	90	0.37	4.71	1.37	
11y 5m	49	75-90	157.6	75-90	0.93	4.08	5.15	
11y 7m	51.2	75-90	158	75-90	6.77	3.79	0.86	0.15
11y 10m	56.2	75-90	160	90	0.28		1.11	
13y 6m	64	90	163	50-75				0.1

Table 4: Anthropomorphic, thyroid hormone, and methimazole dose data for the index patient. Serum levels of fT4, fT3, and TSH were determined with electrochemiluminescence "ECLIA" with a Cobas e 411 analyzer (Roche Diagnostics, Warsaw, Poland). Normal values for fT4 ranged between 0.71 and 1.55 ng/dL, for fT3 between 2.6 and 5.4 pg/mL and for TSH between 0.32 and 5.0 (μIU/mL).

The index patient's younger brother was the second male child born by caesarean section in good condition (10 points on Apgar scale) to a mother treated with l-thyroxin due to hypothyroidism following total thyroidectomy and radioiodine therapy of a hyperthyroid goitrous thyroid (see below). His thyroid hormone levels were assessed at birth due to positive family history for

hyperthyroidism. However, there were no abnormal findings for his first 6 years of life. At six years of age the patient presented with tachycardia and low body mass (body mass: 22 kg (50-75th percentile); height 123cm (75-90th percentile)). Suppressed TSH of <0.01 μIU/mL (ref: 0.32-5.0), fT3 of 8.24pg/mL (ref: 2.6-5.4), fT4 of 2.76 ng/mL (ref: 0.71-1.55) based on ECLIA assay by Cobas, and negative ATG, TPO and TSHR antibodies by ECLIA E170, Roche Diagnostics. Ultrasound showed a normoechogenic, homogenous thyroid with normal volume and excessive flow. Methimazole therapy of 0.5mg/kg/day (taken orally in two doses/day) and propranolol 0.5mg/kg/day (taken orally in two doses/day) was started. Normalization of fT4 (TSH 0.02 μIU/mL (ref: 0.32-5.0), fT4 1.61 ng/mL (ref: 0.71-1.55) ECLIA Cobas, Warsaw Poland) 7 months after the start of antithyroid drug treatment led the treating physicians to decrease dose of methimazole to 0.3mg/kg taken in two doses/day and to stop beta blocker therapy. He is presently 8 years old (height: 138cm- 75th percentile; body mass 32kg- 75-90th percentile)(86). The most recent ultrasound at 7 years of age showed a normoechogenic thyroid with slightly increased blood flow (RL: 2x2.3x3.55 cm, volume: 8.5 mL; LL- 2x2x4.12cm, volume: 8.48 mL; isthmus-0.34 cm). This patient did not experience the thyroid hormone level fluctuations observed in the index patient and was never hypothyroid.

The index patient's mother was diagnosed with hyperthyroidism at six years of age based on physical examination, hormone tests, ultrasound and scintigraphy. TSHRAb were not determined; anti-TPO and anti-Tg were negative, although the assays used are not known. Her father and two siblings also had been diagnosed with hyperthyroidism. For six years she was treated with methimazole (3x5mg) and propranolol (2x20mg). Unfortunately, only limited antithyroid drug dosage information is available. However, her file notes do mention that she required high doses and attempts at reduction of anti-thyroid drugs were not successful. The notes also mention that

large variations of thyroid hormone levels have occurred during treatment although specific information is not available. At twelve years of age she had a subtotal thyroidectomy for an enlarged thyroid gland. At this time the patient had normal TSH and thyroid hormone levels with 5mg 3x/day of methamizole and 20mg 2x/day of beta blocker. Preoperative ultrasound showed enlarged hypoechogenic multinodular goiter with two small nodules (10mm and 15mm) in the right lobe, one nodule in the left lobe (8mm), and a cystic nodule on the boarder of the top of the right lobe and isthmus (55x36mm). Dimensions of the right and left lobe were 51x31mm and 55x32mm respectively. There are no records of the thyroid volume. Scintigraphy showed a blurred lobe structure with a significantly enlarged thyroid, particularly the region of the isthmus which descended 1-2cm behind the sternum. The Tc distribution was uneven, and its highest accumulation occurred in the moderately enlarged right lobe, the upper left lobe and in the isthmus. The large 55x36mm nodule was cold. There are no post-operative findings available. Two years after surgery she was hospitalized for symptoms of hyperthyroidism and her treatment was modified. Unfortunately, the available records do not specify this further. At the age of 24, due to significant compression of the patient's trachea, she was treated with radioiodine therapy. However, there was low I131 uptake by the largest thyroid tumour. The patient declined further surgery following the radioiodine treatment. No data is available for the radioiodine dose. At the age of 30, she had a total thyroidectomy due to an enlarged multinodular goiter compressing her trachea. Preoperative ultrasound findings showed enlarged asymmetric hypoechogenic multinodular goiter compressing the trachea with excessive vascular flow. The largest normoechogenic nodule was found in the right lobe (1.5x1.3cm). The right lobe measured 3.5x3.0x4.5cm, the isthmus measured 0.2cm and the left lobe measured 5.7x4.5x7.0cm. Postoperatively, treatment with 100ug/day of l-thyroxin was started. Her father and affected

siblings (Fig. 5) also underwent total thyroidectomy for hyperthyroidism however no further clinical data are available. No clinical or biochemical adverse events were observed during the treatment of any of the patients. A third child was also born in 2018 and did not show signs or symptoms of hyperthyroidism at the age of one year.

3.3 Methods

Genomic DNA was extracted from EDTA blood samples obtained from the patients and their families. *TSHR* point mutations were investigated by HRM PCR, using primers reported in the study by Eszlinger et al. (69), encompassing exons 9 and 10 (those exons in which all the previously reported constitutively activating *TSHR* mutations were detected) using the LightCycler 480 High Resolution Melting Master chemistry (Roche, Mannheim, Germany) on a LightCycler 480 (Roche, Mannheim, Germany). PCRs to detect *TSHR* point mutations were processed through an initial denaturation at 95 °C for 10 min followed by 55 cycles of a 3-step PCR, including 3 s of denaturation at 95°C, a 10 s annealing phase at 56°C, and a 10 s elongation phase at 72 °C. Thereafter, a high-resolution melting curve was assessed from 75°C to 95°C with an increase of 0.02°C/s and 25 acquisitions per degree. DNA suspicious for *TSHR* mutations in HRM-PCR was subsequently sequenced using Big Dye-terminator chemistry (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions and analyzed on an automatic sequencer ABI 3730xl (Applied Biosystems, Darmstadt, Germany).

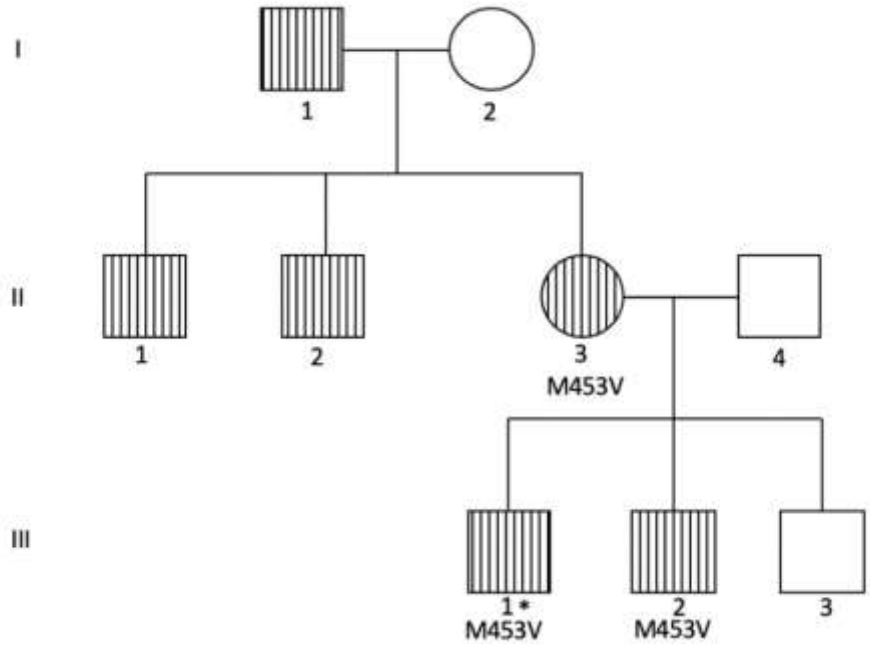

Figure 5: Pedigree for reported family where circles and squares with lines represent history of hyperthyroidism. Non-filled-in individuals did not have symptoms of hyperthyroidism. Index patient identified with *. Individuals with "M453V" have been confirmed to carry the TSHR p.M453V mutation. II/4 has also been tested and is negative for TSHR mutations. Samples from I/1, II/1, and II/2 were not available for molecular testing but the respective family members had documented hyperthyroidism symptoms. III/3 has not shown symptoms and has not had molecular testing.

3.4 Results

3.4.1 Genomic Analyses

In the first case, HRM PCR(89) followed by Sanger sequencing detected a c.1895C>T mutation leading to threonine being substituted by isoleucine (p.T632I) in the index patient. No mutations were identified in either of the parents. As no mutation was detected in the parents and the patient's mutation appeared to have a mutated allele frequency similar to the frequency of the wildtype allele with Sanger Sequencing (~50%), this mutation is categorized as a sporadic germline mutation(2).

HRM PCR followed by Sanger sequencing detected a c.1357A>G; M453V mutation in the two boys and their mother (III/1, III/2, II/3) from the second case. No mutations were identified in the index patient's father. The novel TSHR mutation was evaluated based on SIFT and PolyPhen2 scores to predict whether amino acid substitutions were deleterious (74, 75). The SIFT score for this mutation is 0.01 (deleterious), and the PolyPhen2 score is 0.99 (probably damaging).

3.4.2 Literature Review

The number of generations reported as undiagnosed with FNAH in previous case reports varies since availability of family records and knowledge of family member's health concerns is likely to be a major determining factor. There is only one family where NAH was diagnosed in the first generation (although the case report was published later when the second generation was diagnosed with FNAH) (90).There are 22 families where two generations were described with hyperthyroidism at the time of FNAH diagnosis in the respective index case (56, 79, 91-109). There are a further 13 families with findings reported for 3 generations, and 5 families with findings reported for 4 generations with documented hyperthyroidism at the time of FNAH diagnosis in the respective index case (42, 110-123).

Variable manifestation for the same mutation in the same family, defined as different ages of onset, different symptoms or a lack of symptoms of hyperthyroidism in one or more individuals, has been reported in 29 of 41 FNAH cases (79, 91-93, 95-98, 100, 101, 103-108, 110, 111, 113-116, 118-120, 122-126) (Table 5). In the 13 further cases of FNAH, only one individual is well described or there are no details regarding age of onset, symptoms, and treatment for any patients in their respective report (42, 90, 94, 99, 102, 104, 109, 117, 121, 123, 127, 128).

A comparison of the 29 of 41 FNAH cases in which more than one patient is well described showed variable age of onset and symptoms within families as well as between different families with the same mutation (Table 5). Within the reports for these 29 families there are 110 individuals that have either hyperthyroidism and a suspected TSHR mutation (14 individuals), or a confirmed TSHR mutation (96 individuals). Of these 110 individuals 50 underwent thyroidectomy (5 subtotal followed by total, 21 subtotal or partial, 14 total or near total and 10 that did not specify but are likely total or near total) while only 15 underwent radioiodine treatment (10 in addition to surgery, 5 exclusively)(Table 5).

45 of the 110 individuals had goiter development. Goiter development was present in at least one member of 19 of 29 families, four additional families had slight thyroid enlargements (Table 5). Five families had at least one member where thyroid size was noted to be normal with no information for others while the remaining family had no description of thyroid size for any family members. In summary, thyroid enlargement was present in 24 of 28 families with documented evidence on thyroid size.

Table 5: Inter and intrafamilial variation of symptoms of hyperthyroidism and age of onset of symptoms of hyperthyroidism for families with familial non autoimmune hyperthyroidism where more than one patient is described for the data included in the table. Families were selected according to the TSHR Mutation Database (tsh-receptor-mutation-database.org). "No information" is indicative of a patient for whom there was no mention of the information presented in the respective column.

Reference	Number of described patients	Age of Onset of Hyperthyroidism Symptoms (generation)	Hyperthyroidism Symptoms	Thyroidectomy	RI Therapy	Evidence for Thyroid Enlargement	TSHR Mutation
Oliver-Petit 2017 Clin Case Reports	4	1. Before birth (II) 2. 4 years (II) 3. 8 years (I) 4. <27 years	1. tachycardia, 2. advanced growth 3. advanced growth 4. "hyperthyroid symptoms"	1. total, 34 months 2. total, 5 years 3. total, 27 years 4. total, 27 years	1. no 2. no 3. 27 years 4. no	1. yes, goiter, 4mL (ref: <2.5mL) 2. yes, goiter 5.5mL (ref: <3mL) 3. compressive goiter 4. "huge multinodular vascular goiter"	C672W
Nishihara 2007 Endocr J	6	1. 20 years (IIA) 2. 21 years (IIB) 3. 48 years (IB) 4. no symptoms(IA) 5. no symptoms (IIA) 6. no symptoms (IIB)	1. heat intolerance, palpitation, weight loss 2. heat intolerance, palpitation, weight loss 3. heart failure 4. asymptomatic, subclinical hyperthyroidism 5. asymptomatic, subclinical hyperthyroidism 6. asymptomatic, subclinical hyperthyroidism	1. no 2. no 3. near total, 48 years 4. no 5. no 6. no	1. yes 2. no 3. no 4. no 5. no 6. no	1. mild diffuse goiter 2. mild diffuse goter 3. no information 4. no information 5. no information 6. no information	D617Y
Nwosu 2006 Thyroid	4	1. "youngster"(I) 2. "lifelong" (II) 3. "lifelong" (II) 4. no symptoms (II)	1. sweaty, fast heart rate, increased energy, hyperactivity 2. similar to 1 3. similar to 1	1. no 2. no 3. no 4. no	1. 42 years 2. no 3. no 4. no	1. mildly enlarged 2. enlarged 3. enlarged 4. no enlargement	F621S

			4. asymptomatic, biochemically hyperthyroid				
Biebermann 2001 JCEM	3	1. 3 years (III) 2. 2 years (II) 3. "adolescence" (I)	1. sleep difficulties, hyperactivity, voracious appetite but no weight gain 2. difficulty sleeping, tremor of hand, decreased attention span, prominent eyes 3. no information	1. total, 7.9 years 2. subtotal, 7.6 years 3. subtotal, 15 years	1. no 2. no 3. no	1. enlarged gland 2. no information 3. unilateral goiter	G431S
Nishihara 2010 Thyroid	4	1. 64 years (I) 2. 38 years (II) 3. 30 years (II) (age at evaluation)	1. nodular thyroid lesion 2. asymptomatic 3. asymptomatic	1. no 2. no 3. no	1. no 2. no 3. no	1. unrelated cold thyroid nodule 2. mild goiter 3. mild goiter	E575K
Elgadi 2005 Acta Pediatrica	3	1. 4 years (IIIA) 2. 17 years (IIA) 3. 13 years (IIB) 4. unknown (I)	1. stiffness and pain in lower limbs, one year later includes sweatiness 2. no information 3. no information 4. tachycardia	1. subtotal, 8.4 years 2. no 3. no 4. no	1. no 2. no 3. no 4. no	1. no goiter 2. no goiter 3. no goiter 4. no goiter	G431S
Larsen 2014 Int J Pediatr Endocrinol	3	1. 3 years (II) 2. "always growing up" (II) 3. 16-17 (I)	1. low body weight, failure to thrive, prominent eyes (II) 2. similar to 1 3. tachycardia	1. no 2. no 3. yes	1. no 2. no 3. 18 years	1. mild enlargement 2. mild enlargement 3. no information	G431S
Jaeschke 2014 JCEM	7	1. <18 years (IIA) 2. <36 years (I) 3. <15 years (IIB) 4. 10 years (IIIA) 5. 11 years (IIIA) 6. 8 years (IIIA) 7. 6 months (IIIA)	No information 6&7 asymptomatic with subclinical hyperthyroidism	1. 22 years 2. 36 years 3. 15 years 4. no 5. no 6. no 7. no	1. no 2. no 3. no 4. no 5. no 6. no 7. no	1. no information 2. no information 3. toxic nodular goiter 4. slightly enlarged 5. slightly enlarged 6. no information 7. no information	L665F
Nakamura 2014 Pediatr Res	3	1. 1 month (II) 2. 24 years (I) 3. 6 years (II)	1. tachycardia, poor weight gain 2. headache, fatigue, goiter	1. no 2. no 3. no	1. no 2. no 3. no	1. goiter developed during treatment 2. goiter	M453R

			3. no symptoms, slightly tachycardic			3. goiter developed during treatment	
Supornsilchai 2009 Clin Endocrinol	3	1. 4 months (II) 2. 2 days (II) 3. 1 month (I)	1. low weight, diarrhea 2. craniosynostosis, exophthalmos 3. no information	1. 4 years, subtotal 2.no 3. 8 years, 18 years	1. no 2. no 3. 18, 21, and 28 years	1. goiter 2. no information 3. no information	M453T
Fuhrer 2000 Thyroid	8	1. 4.9 years (IIIA) 2. 21 years (IIA) 3. 9 years (IIB) 4.7 years (IIIB) 5. 5 years (IIIB) 6. 2 years (IIIB) 7. 13 years (IIC) 8. 20 years (I)	1. hyperactive, heat intolerance, hyperphagia with normal weight 2. ND 3. ND 4. asymptomatic 5. asymptomatic 6. ND 7. ND 8. ND	1. no 2. partial, adulthood 3. partial, adulthood 4. no 5. no 6. no 7. no 8. partial	1. no 2. no 3. no 4. no 5. no 6. no 7. no 8. no	1. very small, soft diffuse goiter 2.diffuse hyperplasia 3. no information 4. no information 5. no information 6. no information 7. no information 8. no information	M463V
Lee 2002 J Pediatric Endocrinol Metab	8	1. 7 years (IVA) 2. 5 years 6 months (IVB) 3. 2 years 8 months (IVA) 4. 5 years (IVB) 5. 11 years (IIIA) 6. 20 years (IIIB) 7. 13 years (IIIC) 8. <26 years (I)	1. tremors, weakness, sweatiness 2. asymptomatic 3. sweatiness and hyperactivity 4. tremors, fidgetiness, irritability, weight loss, sweatiness 5. tremors, anxiety, weight loss 6. irritability, weight loss, tremors 7. no information 8. no information	1. no 2. no 3. no 4. no 5. subtotal, 19 years 6. 6 years 7. no 8. subtotal, 26 years	1. no 2. no 3. no 4. no 5. no 6. no 7. no 8. no	1. no goiter 2. no goiter 3. no information 4. no information 5. no information 6. no information 7. no information 8. no information	M463V
Arturi 2002 J Endocrinol Invest	8	1. 14 years (I) 2.17 years (I) 3. 18 years (I) 4.12 years (II)	All affected family members were symptomatic	1. partial, 29 years 2. partial, 40 years	1. yes 2. yes 3. yes 4. no	Goiter in all patients	M463V

		5. 12 years (II) 6. 14 years (II) 7. 14 years (II) 8. 11 years (II)		3. no 4. partial, 17 years 5. partial, 19 years 6. no 7. no 7. partial, 15 years	5. no 6. no 7. no 8. no		
Tonacchera 1996 JCEM B	3	1. 14 years (II) 2. 23 years (II) 3. mother (I) ND	No data	1. no 2. no 3. subtotal	1. no 2. no 3. no	1.goiter 2. goiter 3. goiter	N670S
Tonacchera 1996 JCEM A	2	1. 17 years (II) 2. mother (I) ND	ND	1. no 2. no	1. yes 2. no	1. goiter 2. goiter	N650S
Ferrera 2007 Thyroid	4	1. 6 years (IIIA) 2. 18 years (IIA) 3. 27 years (IIB) 4. <30 years (I)	1. tachycardia and tremors 2. no information 3. no information 4. no information	1. no 2. near total, 35 years 3. total, 40 years 4. no	1. no 2. no 3. no 4. no	1. slight enlargement (5.7mL) 2. multinodular goiter 3. multinodular goiter 4. no information	M463V
Khoo 1999 JCEM	4	1. 7 years (II) 2. 5 years (II) 3. 4 years (II) 4. 38 years (I)	1. heart murmur, tachycardia 2. heart murmur, tachycardia, weight loss 3. tachycardia, thin, small goiter 4. mitral regurgitation	1. subtotal, 21 years 2. subtotal, 20 years 3. subtotal, 20 years 4. subtotal, 41 years	1. no 2. no 3. no 4. no	1. goiter 2. goiter 3. small goiter 4. no information	P639S
Bieberman 1996 J Endocrinol Invest	4	1. 2 months (IIIA) 2. asymptomatic (IIA) 3. adulthood (IIB) 4. adulthood (I)	No information	1. 6 years 2. no 3. adulthood 4.adulthood	1. no 2. no 3. no 4. no	1. no information 2. no information 3. no information 4. no information	R528H

Vaidya 2004 Clin Endocrinol	3	1. 18 months (II) 2. immediately following birth (II) 3. 9 years (I)	1. tachycardia, poor sleep, small diffuse goiter 2. vomiting, poor weight gain 3. weight loss, nervousness, tremor, proptosis, goiter	1. total, 6 years 2. total, 8 years 3. subtotal 13 years, total 19 years	1. no 2. no 3.21 years	1. small diffuse goiter 2. hyperplasia 3. goiter	S505N
Alberti 2001 Eur J Endocrinol	4	1. 5 years (IIA) 2. 7 years (IIA) 2. 15 years (IB) 4. 18 years (IA)	1. hyperactivity, irritability, heart palpitations 2. no information 3. no information 4. no information	1. no 2. total, 9 years 3. total, 16 years 4. total, 18 years	1. no 2. no 3. no 4. no	1. 5.8mL moderately enlarged thyroid 2. large goiter 3. large goiter 4. large goiter	V597L
Guemas 2003 Horm Res	2	1. infancy, since birth (II) 2. asymptomatic (I)	1. tachycardia, accelerated growth, accelerated intestinal transit, hyperactivity 2. normal thyroid function	1. no 2. no	1. no 2. no	1. no goiter by physical examination 2. no information	A428V
Jaeschke 2011 Horm Metab Res	2	1. 10 years old (I) 2. 6 weeks (II)	1. “overt signs” 2. asymptomatic with suppressed TSH	1. no 2. no	1. 14 years x2 2. no	1. no goiter 2. no goiter	M626I
Akcurin 2008 Eur J Pediatr	3	1. infancy (II) 2. 12 days (II) 3. <32 years (I)	1. poor weight gain, diarrhea, little sleep, irritable 2. no information 3. hypertension, restlessness, sweatiness	1. no 2. no 3. total, >36 years	1. no 2. no 3. no	1. 4.37mL (N: <3.5mL) 2. no information 3. TMNG	A485V
Schwab 1996 Exp Clin Endocrinol Diabetes	3	1. 3 years (I) 2. 3 months (II) 3. 3 weeks (II)	1. no information 2. eye deviation, tachycardia 3. weight loss	1. partial thyroidectomy during adolescence, second surgery after age 20 2. no 3. no	1. yes 2. no 3. no	1. “enlarged gland” 2. normal size 3. volume increased under antithyroid treatment	A623V

Claus 2005 Thyroid	3	1. 16 years (III) 2. 18 years (II) 3. <25 years (I)	1. poor school performance, fainting, heat-intolerance 2. no information 3. no information	1. no 2. 34 years, subtotal 3. 25 years subtotal, further surgery date unknown	1. no 2. 38 years 3. no	1. ultrasound showed slightly enlarged gland (19mL) 2. no information 3. TMNG	I568V
Pohlenz 2006 Acta Paediatrica	2	1. 4 months (II) 2. <9 years (I)	1. weight loss 2. no information	1. partial thyroidectomy 9 years 2. no	1. no 2. no	1. no goiter 2. no information	S505R
Karges 2005 J Endocrinol	3	1. 6 months (III) 2. childhood (II) 3. no information (I)	1. hyperactivity, insomnia 2. no information 3. no information 3. no information	1. no 2. 36 years 3. 60 years	1. no 2. no 3. yes, thyroid cancer detected	1. 99th percentile thyroid volume 2. goiter 3. goiter	A593V
Fuhrer 1997 JCEM	2	1. 2 years (II) 2. 12 years (I)	No information	1. no 2. subtotal, 18 years	1. no 2. 25 years	1. goiter 2. goiter	L629F
Fukata et al. 2018	2	1. 26 years (II) 2. asymptomatic	No information	1. no 2. no	1. 28 years 2. no	1. goiter 2. no goiter	
Present family	2	1. 3 years (II) 2. 6 years (II) 3. 6 years (I)	1. tachycardia, weight loss 2. tachycardia, weight loss 3. no information	1. no 2. no 3. total, 30 years	1. no 2. no 3. yes	1. yes 2. no goiter 3. yes	M453V

There is a total of 73 children (<18 years of age) in these 28 families (Table 5). Three of the 73 children (mean age: 3 years old; range 0.1-6 years) had goiter development that was noted to occur during antithyroid drug treatment (95, 103). 29 children (mean age 8 years; range 0-17 years) had goiters present at the time of diagnosis (56, 79, 91-93, 95, 97, 110, 111, 113, 118, 122, 129). A further 10 children (mean age 5.8 years; range 0-16 years) had mild enlargement of the thyroid (93, 96, 100, 105, 116, 119, 122). Seven children (mean age 2.5 years; range 0-7 years) had normal thyroid volumes (91, 95, 96, 115) while 24 children (mean age 6.8 years; range 0-17 years) did not have information available on thyroid size (92, 96, 98, 100, 107, 113-115, 120, 122, 123, 125). In summary there are 42 children with documented evidence of thyroid enlargement of 49 with description of thyroid size.

In previously documented cases of neonatal NAH, patients presented with a spectrum of symptoms, the most common of which were tachycardia, restlessness, poor weight gain and diarrhea. Children with biochemical hyperthyroidism are nearly always frankly symptomatic, especially with adrenergic symptoms (130, 131). To date, there are 20 cases of SNAH in children and 22 families with 25 well described children (<18 years of age) with FNAH(2). Common symptoms found in these patients include tachycardia, restlessness and weight loss (42, 79, 91-97, 106, 110, 111, 113-116, 121, 123, 125, 132-151). In 15 reports (8 cases of SNAH and 7 cases of FNAH) with a documented range of time between onset of symptoms and treatment of 0 to 10 months, advanced bone age was present at 1.3-8.8 times the chronological age. In these 15 cases of NAH in children where advanced bone age is present and duration of symptoms prior to treatment is documented, the correlation coefficient of symptom duration before the start of

antithyroid treatment and bone age/chronological age, excluding patients untreated for >1 year, is 0.0105 (Figure 6).

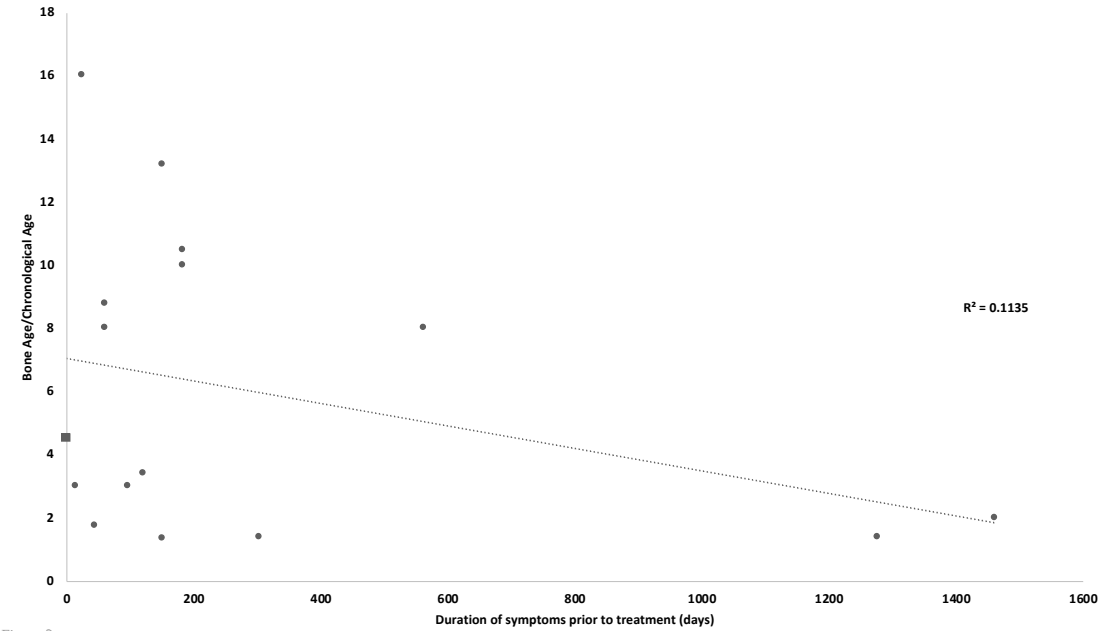

Figure 6: Linear regression analysis of bone age/chronological age and duration of symptoms of patients with this information documented including present case (represented as a square) with correlation coefficient of 0.1135

3.5 Discussion

3.5.1 Case Summaries

In the first study, we describe the case of an asymptomatic neonate with biochemical hyperthyroidism where molecular analysis revealed a TSHR mutation, p.T632I. Current literature identifies this mutation as gain-of-function, causing constitutive activation of the cyclic adenosine monophosphate (cAMP) pathway (60, 152). As this mutation was not present in either parent, it occurred sporadically in our patient, thus our patient's diagnosis is SNAH. This mutation has

previously been described as a somatic mutation in hot thyroid nodules as well as in both a hot follicular thyroid carcinoma and a hot papillary thyroid carcinoma (60, 89, 153).

Additionally, the same nucleotide substitution has been previously described in a case of SNAH with a severe phenotype (132) However, at variance with the case described by Kopp et al. (132), here we describe a patient with the same TSHR mutation in whom hyperthyroidism was diagnosed without any symptoms. Very different clinical manifestations with regards to severity of symptoms and age of onset for hyperthyroidism have previously been described for the same TSHR germline mutations causing neonatal NAH (95, 138, 149).

In the second case, we describe a family previously diagnosed with hyperthyroidism based on thyroid hormone levels, scintigraphy, ultrasound, and physical examination. In previous generations the etiology of hyperthyroidism was not further defined. We have now detected a *TSHR* c.1357A>G; M453V germline mutation in several family members with NAH with complete genotype – phenotype segregation (Figure 5). This is the first time this mutation has been described in association with hyperthyroidism. Based on SIFT and PolyPhen2 scores of 0.01 and 0.99 respectively, we can assume that this mutation has an effect on the protein function. However, functional characterization for this mutation is necessary to finally prove constitutive activity. As the mutation was detected in the mother and her children, this mutation is categorized as a familial constitutively activating germline mutation causing FNAH. Furthermore, one adult male in the first generation and two adult males in the second generation (Figure 5) are also suspected to have a mutation in the TSHR germline mutation based on their available descriptions. Unfortunately, no medical documentation is available for these family members.

The index patient had excessive thyroid growth due to the TSHR germline mutation and increased TSH for two 2 periods of at least 1 month after the first detection of the elevated TSH (Table 4) caused by intermittent inappropriately high doses of methimazole. The initial period of increased TSH was detected after a period of six months without thyroid hormone measurements, the second period after two months without thyroid hormone determination. It is unknown how long the child had increased TSH during these periods of time. The mother also presents with a history of excessive thyroid growth. Unfortunately, due to the limited availability of her medical records, it is not possible to identify whether or not she endured periods of increased TSH. Interestingly, the second child did not have the same fluctuations of thyroid function during treatment and had a much more straightforward antithyroid drug dosage history.

3.5.2 Thyroid enlargement and necessary interventions in NAH

Constitutively activating TSHR mutations have a growth promoting effect on the thyroid (21, 118, 133, 136, 154). 19 of 29 well described FNAH families had goiters (not including slight enlargements which accounts for an additional four families)(Table 5). Whether these goiters were due to later diagnosis or treatment is unknown. Of 49 children with description of thyroid size, 32 were reported to have goiter development, another 10 with mild thyroid enlargement. In three children with goiter noted to develop during treatment, the cause of the goiter could be analogous to that of our index patient (95, 103). Some cases may have had no evidence of thyroid enlargement due to early diagnosis and evaluation. However, for 29 cases of children who clearly developed goiter prior to diagnosis, constitutive activity of the TSHR may be the sole driver of growth (79, 91-93, 95, 97, 110, 111, 113, 118, 122, 129).

The degree of constitutive activity of the G-protein coupling profile of the respective TSHR mutation may also play a role in the development of goiter as was shown in a case of sporadic NAH due to TSHR mutation with only slightly increased constitutive activity. This led to a late onset of symptoms at the age of 69 at which time the patient had a 102ml goiter, likely from a lifetime of slightly increased TSHR activity (155). The two individuals of the family in this report where high TSH had a likely aggravating role in addition to the constitutive TSHR activity argue for early total thyroidectomy in this family. The second child was diagnosed at 6 years of age, as was his mother, at variance with the index patient who was diagnosed at 3 years. However, the mother did not have a total thyroidectomy until the age of 30 and neither child has undergone surgery to date.

These cases give strong support for the necessity of identifying mutations in the TSHR in patients with difficult to treat hyperthyroidism, especially when it is present across generations in a family in order to diagnose and treat FNAH. Had the male in the first generation been properly diagnosed, all three patients described above, as well as the two males in the second generation, could have received total thyroidectomy at an earlier time point. This would have been especially beneficial to the mother (II/3) who underwent two surgical interventions and radioiodine treatment as well as ineffective metamizole therapy. Early diagnosis and treatment by total thyroidectomy is also supported by cases with severe consequences of hyperthyroidism such as heart failure (124), and by cases where repeated surgeries were needed after a subtotal thyroidectomy did not alleviate the patient's symptoms (42, 91-93, 95, 116). Unfortunately, late diagnosis of FNAH is not uncommon with 18 of 41 published families of FNAH presenting with three of more generations with hyperthyroidism at the time of diagnosis. Knowledge of familial TSHR germline mutations allows

for earlier hyperthyroidism and goiter relapse risk assessment to determine the appropriate therapy for patients with FNAH. Patients with FNAH require total thyroidectomy as antithyroid drug therapy would have to be maintained indefinitely since remission of hyperthyroidism cannot be expected. Moreover, antithyroid drug therapy of FNAH is complicated by variations of the severity of hyperthyroidism, often difficult to adapt antithyroid drug treatment, especially during childhood and is subject to compliance problems and adverse events. Remission of hyperthyroidism does not occur in FNAH and total thyroidectomy is necessary (156).

3.5.3 Variability in NAH

Variable onset of symptoms, variable symptoms at manifestation of hyperthyroidism and variable treatments required within the same family with a TSHR germline mutation has been described in all 29 FNAH case reports with detailed clinical information for more than a single patient (Table 5). The number of cases with variable presentation is likely higher due to only one individual being well described in the further 12 FNAH case reports. As expected, in these 29 families, surgery and radioiodine treatment for patients within the family are not consistent (Table 5) supporting the assertation that there is true variability in the phenotype, not only in the reported symptoms/availability of data for onset of symptoms. However, there are recurrent findings, on average 45% of family members underwent surgery in each family while only 15% of family members undergo radioiodine treatment. These case reports may not be representative of the true course of treatment over a patient's lifetime with FNAH. Patients not undergoing thyroidectomy at the time of publication does not preclude them from doing so at a later date. While the index patient and his brother in our case report have not undergone total thyroidectomy they likely will in the future, and it has been proposed to their parents by their physician.

Variation of age of onset, symptoms at onset, and treatment has also been noted in different families with the same mutation (118, 152). For example, for M463V there are four families, age of onset varies from 2-30 years, some patients are asymptomatic while others exhibit severe symptoms including tachycardia, weight loss and tremors. Two of these families have previously been compared (118). The treatment of these patients also varies, some symptomatic patients were not treated while some patients required both surgical intervention and radioactive iodine treatment. Different degrees of in-vitro constitutive activity or different G-protein coupling profiles of the respective TSHR mutations may be one explanation for variation of age of symptom onset, symptoms and treatment between families with different TSHR mutations (31, 128, 157). Table 2 demonstrates that age of onset can vary from before birth to 64 years of age, that patients can be asymptomatic or present with life threatening symptoms and may require limited medical treatment or multiple surgeries (45%) and radioiodine treatment (15%). However, variations between individuals in a family is evidence for the involvement of factors other than constitutive activity or G-protein coupling profile of the respective TSHR mutation. These factors could include β-arrestin induced desensitization and down regulation of the receptor or variations in other signaling molecules as well as iodine intake and genetic background of individuals (23, 24, 158, 159). Our case report supports previous findings suggesting that the phenotype of this disease is related to more than just the in-vitro constitutive activity of the respective TSHR mutation (118, 125).

3.5.4 Advanced Bone age

A strong example of phenotype variability and the unknown variables in NAH is found in the first case. This case is distinguished from all other cases of neonatal NAH by the documented presence

of advanced bone age before onset of symptoms. Had her mother not been treated with levothyroxine during her pregnancy, our asymptomatic patient would not have been evaluated for hyperthyroidism based on heart rate (within normal range) and lack of other symptoms of hyperthyroidism, despite her advanced bone age, which may have gone undetected.

Advanced bone age is indicative of untreated hyperthyroidism, early in the pathogenesis of NAH. Advanced bone age is an important concern for children with hyperthyroidism as it impacts adult height and the effects of untreated SNAH hyperthyroidism are not reversible (40, 160, 161). It is not known if the induction of advanced bone age is dependent on the duration of hyperthyroidism.Untreated hyperthyroidism can lead to complications in children and neonates such as craniosynostosis, tachycardia, mental retardation (162). There are 26 cases of children with NAH and advanced bone age in 10 families and 13 sporadic cases. Only 15 of these 26 cases have quantified advanced bone age and documented time of symptoms prior to treatment (shown in Table 1). However, of these 15 cases, the correlation coefficient of symptom duration before the start of antithyroid treatment and bone age/chronological age, excluding patients untreated for >1 year, is 0.0105. Although these data are anecdotal, this suggests an absence of correlation between these variables (Figure 6).

Although there is considerable inter and intra-rater variability in bone age determination and different methods for the evaluation of bone age were used, these findings argue that neither the duration of symptoms prior to treatment nor the age at onset of symptoms, two factors which are likely to be the best possible indicators for the duration of untreated hyperthyroidism, explain the

degree to which advanced bone age is present. These findings are in line with the finding of this case report with advanced bone age despite a presumed short duration of hyperthyroidism.

TSHR has been demonstrated to be expressed in both osteoblasts and osteoclasts, albeit with cAMP independent pathways (163). While some studies have proposed that the effect of thyrotoxicosis on the bone is due to increased thyroid hormone rather than decreased TSH, the role of the TSHR in bone is still uncertain (164-166). The degree of hyperthyroidism may be indicative of one aspect of TSHR signaling whereas the advanced bone age/bone phenotype could also be the result of an alternative pathway based on coupling of TSHR to all four G protein subfamilies (167), some of which are yet to be fully explored, particularly in bone.

NAH patients do not respond well to antithyroid drug treatment and remission of hyperthyroidism is rarely achieved. This was demonstrated early in our case as the patient needed several dose increases which resulted only in transient periods of normalization of thyroid function. For this reason, thyroidectomy is the treatment of choice for NAH (156). Detection of a TSHR germline mutation and verification of the diagnosis of NAH provides a justification for early total thyroidectomy. Less complete surgeries are associated with frequent relapses (156).

3.5.5 Conclusion

In summary, we first report a case of SNAH due to a previously characterized constitutively activating TSHR mutation. This case is unique in that NAH was detected prior to the onset of symptoms, yet bone age was already advanced. We also report a case of FNAH due to a novel TSHR mutation. Our case reports and our review of the literature illustrates the variability of the

NAH phenotype, a compelling argument for the early diagnosis and appropriate treatment with early total thyroidectomy for FNAH and SNAH. This is necessary in order to avoid predictable, unnecessary complications such as multiple surgeries, impaired bone development and no remission of hyperthyroidism after treatment with methimazole for index patients and for future generations as well.

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CHAPTER 4: TSHR MUTATION DATABASE UPDATE

4.1 Relevance

Based on the literature reviews accompanying the case reports in the previous two chapters, the effects of TSHR signaling are evidently variable. For this reason, it is important to have a method of tracking and data collection to ensure further analysis can be done as the number of documented cases for the rare disorders associated with the TSHR increases.

4.2 Introduction

Somatic mutations have been found in up to 84% of single toxic thyroid nodules as well as in toxic multinodular goiters (168-172), our tNGS study above found a 92% of HTA to contain a TSHR mutation(1). The prevalence of inactivating TSHR mutations has been reported with much variability. Inactivating familial or sporadic TSHR mutations were reported in 13.7% Japanese patients with congenital hypothyroidism: 4.3% with biallelic mutations, and 9.4% with monoallelic mutations (173) whereas a population-based Italian study found 1/16 (6.3%) and a Finnish study found 2/26 (7.7%) congenital hypothyroidism patients to have monoallelic TSHR mutation (174, 175). Mutations have also been found in patients with uncommon conditions such as hyperfunctioning thyroid carcinomas (89, 153, 176-187) and gestational thyrotoxicosis, a transient form of thyrotoxicosis during pregnancy due to a mutant thyrotropin receptor hypersensitive to human chorionic gonadotropin (99).

The TSHR mutation database, established in 1999, documents the clinical findings of patients with TSHR mutations and the functional characterization, the degree to which TSHR mutations activate or inactivate the TSHR (188). The database has been updated in 2003 (189), and in 2012 (44).

During the current update, finalized in August 2019, the database was relocated to a University of Calgary server. Its website remains the same (tsh-receptor-mutation-database.org).

4.3 Additions to the database

Five years after the last update, the current database now contains all clinically occurring TSHR mutations published up to August 2019. New entries for 35 novel mutations (7 activating and 28 inactivating) and 72 new cases for previously described mutations (41 activating and 31 inactivating) were added to the TSHR mutation database. A total of 107 new entries were subdivided into 4 categories:

1. Constitutively activating TSHR germline mutations: 5 novel mutations and 12 new cases of previously described mutations
2. Constitutively activating somatic TSHR mutations: 2 novel mutation and 25 cases of previously described mutations
3. Constitutively activating somatic TSHR mutations in hyperfunctioning thyroid carcinomas: 4 cases of previously described somatic gain of function mutations in hyperfunctioning thyroid carcinomas
4. Inactivating TSHR germline mutations: 28 novel mutations and 31 new cases of previously described inactivating TSHR germline mutations.

It is important to note that 28 novel mutations added to the database have not been functionally characterized, this is indicated in each mutation's preview accessed from the list page (https://tsh-receptor-mutation-database.org/list.html), or on the details page for each mutation. While most of

the novel mutations associated with a hyperfunctioning nodule have been functionally characterized (5/7), only a small number of novel inactivating mutations (2/28) have functional characterization data available. One study documenting a novel mutation associated with a hyperfunctioning phenotype justified this by citing a study showing a different amino acid exchange at the same position associated with activating function (145) while large scale population studies did not address the lack of functional characterization (190). We would like to emphasize the importance of functional characterization of new TSHR mutations (152, 157).

Constitutively activating mutations are generally found in the transmembrane domains, with a disproportionate number found in the sixth transmembrane domain. These are generally point mutations with the exception of 3 constitutively activating deletion mutants (191-193). Inactivating mutations are found throughout the receptor and more commonly include deletions, insertions, truncation and splicing (80, 81, 129, 173, 194-204).

4.4 Geographic origin of reported mutations

The previous database had 200,000 hits worldwide since its debut in 1999. Currently, there is a dearth and an underrepresentation of North American patients with TSHR mutations in this database. Interestingly, much of the data in the TSHR mutation database comes from studies in Europe. Asia (particularly Japan) is the second most active continent with published clinical reports on TSHR mutations (Figure 7, Figure 8). These data are probably subject to ascertainment bias; however, it is of interest to see which countries are presently screening for TSHR mutations. Interestingly, some mutations have been reported across different continents (North America, Europe, Asia). There are 14 activating germline mutations described more than once, 6 of these occur only within a single continent (4 in Europe, 2 in Asia), the further 8 mutations have been

described in multiple continents (Table 6). Certain inactivating germline mutations referenced in the database have a higher incidence in particular countries or continents, for example R450H has been described 20 times, 16 of which were in Japan with two further incidences in Italy and one in China whereas P162A has been described 22 times, 16 in Italy, 4 elsewhere in Europe, once in USA and once in Thailand.

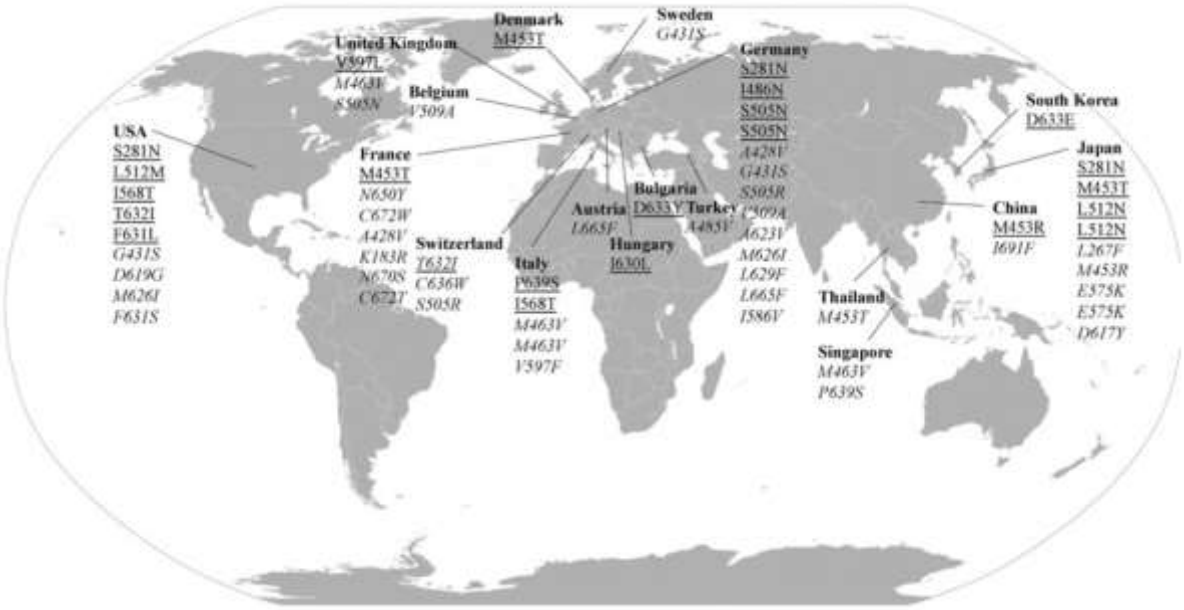


Figure 7 Location of clinician authors caring for respective patients for published cases of activating TSHR mutations where there are 39 familial (italics) and 23 sporadic (underlined) cases of activating germline mutations at the time of the database update publication.

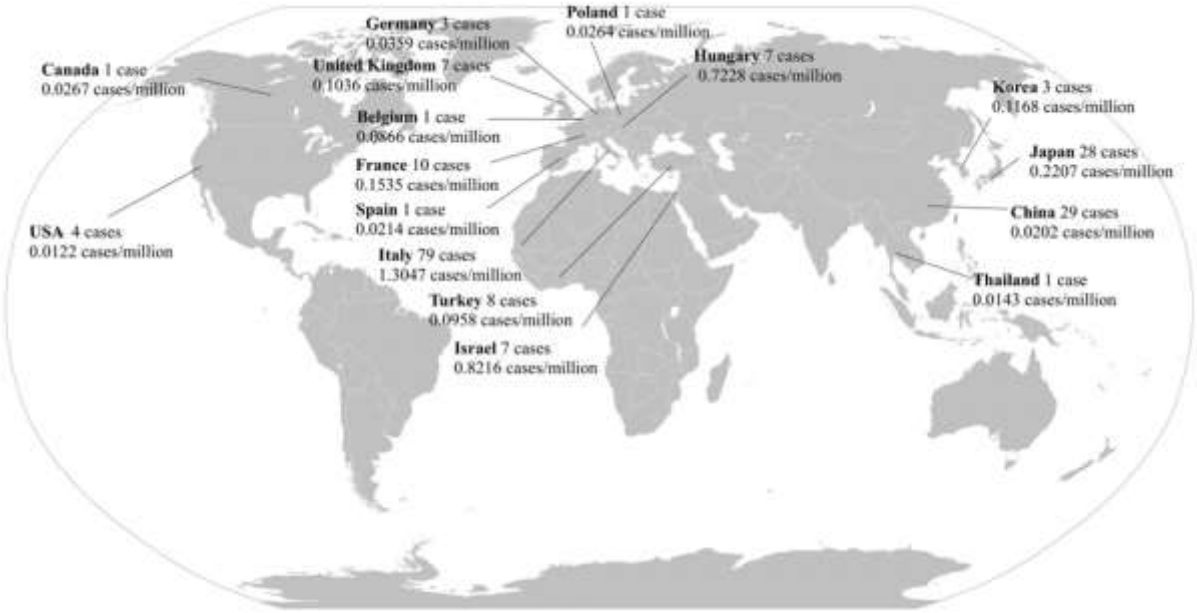


Figure 8 Estimated number of published cases and cases per million of inactivating germline TSHR mutations based on location of clinician authors caring for respective patients. Population statistics from 2019 Revision of World Population Prospects by United Nations Population Division.

Table 6 Countries of origin for patients with constitutively activating germline mutations described more than once

Mutation	*Location 1*	*Location 2*	*Location 3*	*Location 4*
S281N	Germany	Japan	United States	
A428V	France	Germany		
G431S	Germany	Sweden	United States	
M453T	Denmark	France	Japan	Thailand
M463V	Italy	Italy	Singapore	United Kingdom
S505R	Germany	Germany	Germany	United Kingdom
V509A	Belgium	Germany		
I586T	Japan	United States		
L512M	Japan	Japan		
E575K	Japan	Japan		
M626I	Germany	United States		
T632I	Switzerland	United States		
P639S	Italy	Singapore		
S505N	Germany	Germany		

4.5 Mutations described in thyroid carcinoma

In this update, we have included a new category for TSHR mutations described in cases of hyperfunctioning thyroid carcinoma. The following mutations have been described in cases of thyroid carcinoma presenting as a hot thyroid nodule: I486P (180, 181), M453T (177-179), L512R (182), I568T (183), T620I (184), A623S (185), F631I (186), T632A (153), T632I (89, 153), D633H (187), D33Y (186), and L677V (176), I568P (205), F631L (205), I630L (205). Clarification if or how these mutations could contribute to the development of thyroid carcinoma is debated and requires further comprehensive genetic analysis of the published TSHR mutation positive carcinomas along with further cases of thyroid carcinoma presenting as a hot thyroid nodules without mutational analysis. The cases described in our database highlight the need for further investigation of the role of TSHR in thyroid carcinoma, especially considering that the increased malignancy risk for TSHR mutation positive hot nodules in children does not appear to be associated with most frequent mutations such as RAS, BRAF, PAX8/PPARG and RET/PTC mutations (89).

4.6 Webpage Changes

While the functionality of the website remains mostly unchanged, new additions include a summary homepage, and an updated prevalence page (https://tsh-receptor-mutation-database.org/prevalence.html) that reflects the number of cases rather than the number of publications. In order to more accurately estimate prevalence of specific TSHR mutations, cases published more than once were removed by comparing samples in non-case reports to all other publications with overlapping authors. Duplicate entries were added to the mutation reference list but did not contribute to the prevalence calculation.

To speed up database access, we have built a website using LAMP technology (Linux, Apache MySQL, and PHP), one of the most popular and fastest ways to generate web pages. The TSHR mutation database is installed as one of the locus specific HUGO mutation databases (206) and can be accessed via https://tsh-receptor-mutation-database.org. Ralf Paschke continues to serve as curator of the database. The database will be continuously updated.

4.7 Conclusion

New additions to the webpage include a summary, and an updated prevalence page that accounts for mutations and / or cases published more than once. As of July 2020, there are 25 cases of SNAH, 41 families with FNAH, 21 cases of hyperfunctioning thyroid carcinomas, and 202 families or sporadic individuals with hypothyroidism or TSH insensitivity with inactivating TSHR mutations. This database allows for rapid validation of patient TSHR mutations causing hyper- or hypothyroidism or insensitivity to TSH.

CHAPTER FIVE: FUTURE DIRECTIONS FOR TSHR'S ROLE IN CARCINOMA

5.1 Relevance TSHR mutations have been found in 21 cases of hyperfunctioning carcinoma (2). While the consequences of benign constitutively activating TSHR signaling has been thoroughly explored in this thesis, the role of TSHR in carcinoma has not yet been addressed. It was originally part of this project to explore the role of TSHR in thyroid carcinoma using a TSHR KI mouse model; however analysis of the molecular and phosphoproteomic profile was put on hold due to lab closures caused by COVID-19. Additionally, human hot thyroid carcinoma samples were collected for genomic investigation as well as planned RNA-seq. In this final chapter before overall conclusions, the background for this project and the preliminary data that is available thus far will be explored.

5.2 Introduction

To understand the role of TSHR signaling in the development of hyperthyroidism and thyroid growth, our group generated a knock-in (KI) mouse model harboring a patient-derived TSHR D633H mutation (207). This mutation has been reported eight times: seven times in hot thyroid adenoma and once in thyroid carcinoma (41, 63, 169, 170, 187, 208-212). Interestingly, this is one of the few activating mutations with a proposed molecular mechanism of TSHR activation: A specific H-bonding network formed between the central regions of transmembrane domain 6 and transmembrane domain 7 which has been proposed as essential for maintaining inactivation of the TSHR (208). For the first time, our group found that in a transgenic mouse model non-autoimmune hyperthyroidism (NAH) is not as stable as expected but rather a dynamic condition involving age, sex and *Tshr* allele-dependent compensatory mechanisms (207). However, most interestingly, our

data strongly suggest that a permanently active TSHR can lead to the transformation of thyrocytes into cancer cells.

After 12 months, PTCs occurred in both heterozygous (HEZ) and homozygous (HOZ) TSHR D633H KI mice. However, while PTCs were found in 88% of HOZ females and 80% of HOZ males, PTCs were only found in 30% of HEZ females, and 20% of HEZ males. These findings form the basis for the further investigation of this model. We propose that the mechanisms behind PTC development is the same for both HOZ and HEZ as the sex differences remain the same. Thus, we plan to focus on HOZ animals, despite the TSHR mutation being HEZ in humans. The differential gene expression and protein levels will be more pronounced in HOZ mice, allowing for detection of more nuanced responsible signaling pathways and protein expression levels (207).

While the majority of PTCs in humans are typically characterized by a permanently active MAPK signaling due to mutations in *BRAF* or one of the *RAS* genes (213, 214), no such mutations were found in the PTCs of TSHR D633H KI animals (207). However, increased ERK1/2 and phospho-ERK1/2 staining was found in the mice harbouring PTC (207). Together, this suggests involvement of the TSHR through activation of MAPK, in the development of a subset of PTC that are hyperthyroid. There are also instances in the database of HTCs without known oncogenic mutations. In parallel with the mouse project, we set out to collect human HTC (hot thyroid carcinoma) samples for further analysis.

TSHR signaling via cAMP is increased by the D633H KI mutation, as evidenced by the 5.4 fold increase in basal cAMP signaling for the TSHR (207, 208, 215). cAMP signaling has been shown

to activate MAPK signaling (27, 28). As this model does not have common mutations that would activate MAPK such as BRAF and RAS, it is reasonable to propose that the increased p-ERK staining seen in this model is a result of TSHR stimulation of MAPK, albeit potentially in collaboration with an additional mutation which would be infrequent in PTCs (207, 216, 217). While the known increased mutational risk in the C57BL/6J mice[5-7] could suggest that PTC development may require more than constitutive TSHR signaling (218), different mutations result in different carcinoma subtypes and our mouse model has a consistent carcinoma phenotype distinct from the BRAF KI model. The strong genotype-phenotype correlation with deceased carcinoma found in heterozygous mice and none found in wildtype mice suggests that this carcinoma development is much more than an artefact due to the model's propensity to further mutations and that TSHR plays a critical role in carcinoma development. Moreover, in a previous WES analysis of HNs we have demonstrated the absence of any mutations found in PTCs (219).

In summary, these data impressively demonstrate a key role of the TSHR-Gsα-cAMP pathway in PTC initiation and support the role of TSHR signaling in thyroid carcinoma development. The respective responsible signaling pathway(s) and the degree to which the TSHR is involved will be analyzed through exclusion or identification of additional mutations and exploration of the subsequent phosphoproteome changes. Many cancers have associated dysregulated kinase signaling whether this is through kinase overexpression, mutations in kinase genes, or defects in counter regulatory mechanisms (220, 221). As protein phosphorylation is a key regulator for numerous cellular processes, this aberrant signaling is often a driver for the cancer, rather than a consequence. Knowledge of kinase signaling in cancers is beneficial since activated kinases can be targeted with small molecule inhibitors for chemotherapy (222). While genomic changes such

as a TSHR mutation in a carcinoma without any other driver mutations are interesting, it is important to understand the consequences in signaling in order for this information to inform treatment and the etiology of thyroid cancer in general.

5.3 Methods:

5.3.1 Generation and Sacrifice of TSHR D633H KI Mice

Generation of homozygous TSHR D633H KI mice to be analyzed at 12 months began in August 2017 in Turku, Finland. Generation for those to be analyzed at 2 months begun in June 2018. Both groups of mice were sacrificed, and thyroids harvested the tissues from August to December 2018.

5.3.2 Physiological Characterization and Tissue Collection of TSHR D633H KI Mice

For 10 mice from each group (separated by age [2/12months], genotype [HOM/WT]and sex [M/F]), Body weight, body length, tail length, and thyroid weight was recorded at sacrifice. 1mL of blood was collected, and serum separated for a mass analysis of levels of serum TSH and free T3 and free T4. Each lobe of the thyroid was preserved separately, one as formalin fixed paraffin embedded (FFPE) blocks that can be sectioned in the future, and one lobe was flash frozen in liquid nitrogen. Additional muscle tissue was collected and frozen in liquid nitrogen from two homozygous mice of each sex.

5.3.3 Immunohistochemical Characterization of TSHR D633H KI Mice

H&E staining of one slide was performed on all groups of mice (12 and 2 month old, females and males, homozygous and wildtype). Following H&E staining, diagnosis of PTC was be confirmed by pathologist, Dr. Moosa Khalil (pending results for males).

5.3.4 Correlation Analysis

To qualitatively account for PTC presence in H&E stained F HOZ slides, values of 0, 1, 2 and 3 were assigned to each sample where 0 meant no PTC, 1 meant <40%, 2 meant roughly half PTC (40-60%) and 3 meant >60% PTC. These values were compared to the respective values for TSH, tT4 and thyroid weight.

5.3.5 Human Hot Thyroid Carcinoma Samples

Nine hot thyroid carcinoma (HTC) samples were analyzed. Details for the samples and pathologist assessment can be found in Appendix B. These samples were requested based on the characterization suggesting the sample is a hot carcinoma and not two separate entities (euthyroid or hypothyroid carcinoma and hot nodule) in the form of scintigraphy, ultrasound, and histological findings. These samples, preserved as formalin-fixed paraffin-embedded (FFPE) blocks, have been sectioned into slides and sent to us from numerous research laboratories. All samples were reanalyzed by our pathologist collaborator, Dr. Moosa Khalil. DNA was extracted from all samples using Qiagen AllPrep FFPE Kit (Qiagen, Hilden, Germany)

5.3.6 Genomic Analyses of Human HTCs

Extracted DNA was run through multiplex polymerase chain reactions (PCR) with primers specific to the ThyroSPEC™ panel. The amplified sites include 140 known thyroid cancer associated point mutations and gene fusions such as BRAF, RAS, RET/PTC and PAX8/PPARG. Afterwards, PCR products underwent iPlex primer extension, in which terminator dideoxy nucleotides arrested extension at variant allele sites. MALDI-TOF mass spectrometry (MassARRAY) was performed

on the iPlex extension products, as defined by the ThyroSPEC™ panel. Thereby, a profile of the 140 thyroid cancer related mutations was generated for each HTC sample.

TSHR point mutations were investigated by HRM PCR, using primers reported in the study by Eszlinger et al. (69), encompassing exons 9 and 10 (those exons in which all the previously reported constitutively activating TSHR mutations were detected) using the LightCycler 480 High Resolution Melting Master chemistry (Roche, Mannheim, Germany) on a LightCycler 480 (Roche, Mannheim, Germany). PCRs to detect TSHR point mutations were processed through an initial denaturation at 95 °C for 10 min followed by 55 cycles of a 3-step PCR, including 3 s of denaturation at 95°C, a 10 s annealing phase at 56°C, and a 10 s elongation phase at 72 °C. Thereafter, a high-resolution melting curve was assessed from 75°C to 95°C with an increase of 0.02°C/s and 25 acquisitions per degree.

DNA suspicious for TSHR mutations in HRM-PCR was subsequently sequenced using Big Dye-terminator chemistry (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions and analyzed on an automatic sequencer ABI 3730xl (Applied Biosystems, Darmstadt, Germany).

TSHR point mutations in position c.1458 and c.1459 were detected by pyrosequencing using self-designed primers and the following PCR conditions as described in Eszlinger et al. 2014 (89): PCRs were processed through an initial denaturation at 95°C for 15 minutes followed by 42 cycles of a 3-step PCR, including 20 seconds of denaturation at 95°C, a 30 seconds annealing phase at 60°C, and a 30 seconds elongation phase at 72°C, followed by a final 5 minutes extension phase

at 72°C. PCR products were immobilized to streptavidin sepharose beads and single stranded DNA was prepared allowing subsequent annealing of the sequencing primer to the template DNA. Then, the primed single-stranded DNA was released from the streptavidin surface and transferred to a PyroMark Q24 (QIAGEN) for pyrosequencing.

5.4 Results:

5.4.1 Morphology of second generation of TSHR D633H KI mice

TSH is significantly lower in KI males (P<0.01) and females (P<0.001). Body weight is significantly lower in female KI mice than WT (P<0.01). Thyroid weight is significantly higher in both male KI (P<0.001) and female KI (<0.001) (Figure 9).

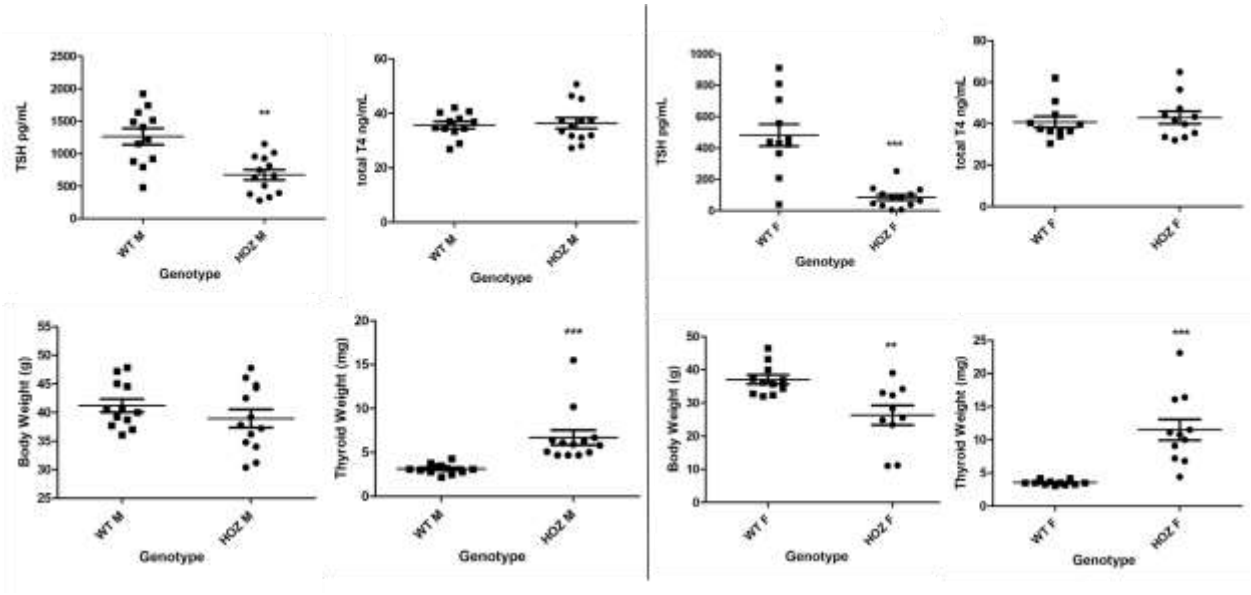


Figure 9: TSH, total T4, body weight and thyroid weight for TSHR D633H KI 12 month old mice with males on the left and females on the right. (** P<0.01, ***P<0.001).

5.4.2 Presence of Carcinoma in Mice

Nine of eleven female HOZ KI TSHR D633H mice have PTC present.

5.4.3 Correlation of morphological variables with PTC

Neither TSH, total T4 or thyroid weight was correlated with the occurrence of PTC. (Correlation coefficients of 0.126, 0.002, and -0.15 respectively).

5.4.4 Human HTC Results

Known cancer-associated mutations were found in 4/9 samples by ThyroSPEC. A further two samples were suspicious for BRAF mutations, and a further sample was both suspicious for BRAF and confirmed NRAS (Table 7). TSHR mutations were detected in 4/9 of the human hot thyroid carcinoma samples (Table 8).

Table 7: ThyroSPEC results for 9 human hot thyroid carcinoma samples, sample details available in Appendix B. Suspicious mutations were below the threshold for ThyroSPEC however were consistent across multiple wells as called by Paul Stewardson. Mi: micro; fv: folliculat variant

	HTC 1	HTC 2	HTC 3	HTC 4	HTC 5	HTC 6	HTC 7	HTC 8	HTC 9
Histology	mifvPTC	FTC	PTC	fvPTC	miPTC	PTC	PTC	PTC	PTC
Confirmed mutations	none	BRAF; HRAS	TP53; EIF1AX	HRAS; TSHR	NRAS	none	none	None	TSHR IDH1
Suspicious mutations					BRAF	BRAF	BRAF		

Table 8: HRM & Sanger Sequencing Results for 9 human hot thyroid carcinoma samples. Italicized results were already published.

	HTC 1	HTC 2	HTC 3	HTC 4	HTC 5	HTC 6	HTC 7	HTC 8	HTC 9
TSHR mutation by Sanger Sequencing	none	none	none	M453T	none	none	C672T	C672T	*M453T*

TSHR by Pyrosequencing (M453T or M453V)	none	none	none	M453T	none	none	none	none	ND

5.5 Next Steps

5.5.1 Whole Exome Sequencing

WES is the preferred method of determining single nucleotide variants, indels and will show all DNA modifications in the exome, even those that may not result in large scale gene expression changes (223). WES will be performed on the thyroid tissue from 5 homozygous female TSHRD633H KI, and 3 WT females since female mice have a more prominent phenotype. For each sample, DNA has been extracted from fresh frozen thyroid lobes using the AllPrep DNA/RNA/Protein kit (Qiagen) according to the manufacturer's protocol. 100ng DNA per sample will be submitted to the Core Sequencing Facility for WES on a Illumina NextSeq 500 instrument. For WES done at the CHGI, basic bioinformatics analysis is included in the sequencing cost.. If any mutations are found, they will be validated with Sanger or Pyrosequencing based on mutated allele frequency. In case of a repetitive mutation pattern, the additional female mice and all 12 mo male HOZ mice will be screened for any mutations found in the female WES cohort using pyrosequencing

We do not anticipate the dilution of thyroid carcinoma with normal surrounding thyroid tissue causing mutations to be overlooked since the proportion of PTC tissue in the previous generation of mice was 50% and we are planning for 150X coverage. A coverage of 52X is adequate under ideal conditions to detect mutations in a sample with only 20% tumour (224) therefore we are confident we will detect relevant mutations in this analysis.

If the additional mutations found are not in known genes, we will attempt to understand the role of these mutations through paired phosphoproteome analysis. Based on variable percentage of PTC found across mice, it is likely that there are numerous secondary mutations. Grouping samples according to their mutational profile and assessing the phosphoproteome results may help to elucidate the role of any unknown mutations.

5.5.2 Phosphoproteome Analysis

The preferred methodology for the elucidation of pathways and proteins responsible for PTC development in TSHR D633H KI mice is phosphoproteomic evaluation of these samples as this allows us to determine which proteins have been translated and phosphorylated compared to their wild type counterparts. Phospho-proteomics looks at phosphorylated proteins from tissue using technologies such as mass spectrometry (MS) for the identification and quantification of protein phosphorylation sites (225).This information will allow us to elucidate the mechanism of PTC development in these mice without any known PTC driver mutations. Since our primary goal is to determine the activated pathways, it is essential that we use a methodology that will help eludicate these. While RNA-seq is a superior methodology to other transcriptomic methods such as microarrays (226), proteomics as compared to transcriptomics gives information regarding the current levels of proteins, and in our case with phosphoproteomics, the state of phosphorylation of these proteins. Direct comparison of transcriptome and proteome coverage indicates that only a part of the dysregulated mRNAs are also dysregulated on the proteome level (G. Moran et al personal communication). While changes detected in the transcriptome require validation from RT-PCR and western blots to ensure that the change occurs in protein as well as RNA,

phosphoproteomics results are indicative of the true state of proteins in the thyroid. Phosphoproteomic data combined with RNA-seq data would of course be the most beneficial as it would allow us to differentiate between changes due to translation or due to other factors such as degradation and to differentiate between increased phosphorylation and decreased dephosphorylation of proteins (227). In Mertins et al's 2016 study, it was found that proteins and phosphosites quantified in breast tumors were generally in agreement. For those that were not, the proposed accountable factor was the higher sensitivity of phosphorylation to these sites relative to MS analysis alone (228)

Signaling pathways involved in the malignant transformation of TSHR D633H KI mice will therefore be identified by phosphoproteomic profiling of PTC of 12-month-old homozygous and wildtype mice to elucidate the role of TSHR mutation induced signaling in a carcinoma with or without any other driver mutations. This approach is poised to unravel for the first time in vivo mechanisms of transition from thyroid adenoma to thyroid carcinoma and inform diagnosis, prevention and treatment and the etiology of thyroid cancer in general. After identifying phosphoproteins and signaling pathways of interest, immunohistochemistry of 2-month mice will determine whether molecules of interest from these signaling pathways are present in 2 month old KI mice to establish whether these changes begin before the development of PTC. This will also be correlated to the timing of the appearance of secondary mutations.

Phospho-proteomic analysis will be performed on 8 HOZ and WT mice of each sex at 12 months of age. Randomly selected lobes from each mouse that were removed and flash frozen in liquid nitrogen will be used for analysis. Total protein will be extracted from ½ lobes sent to the Moran

lab. Protein will be purified and separated using a LC-MS/MS, an instrument with a combination of mass spectrometry and liquid chromatography (229). Data will be analyzed as previously described using MaxQuant software and subsequently compared to the SwissProt database (230, 231). This will take place in collaboration with Dr. Michael Moran and the protein core facility at the Program in Molecular Structure and Function at the Hospital for Sick Children in Toronto, Ontario. Although this experiment will result in global phosphorylation patterns, a focus of our data analysis will be on thyroid specific transcripts, proliferation markers, and proteins related to specific signaling pathways.

5.6 Discussion

The morphological data available so far indicates a relatively consistent phenotype among TSHR D633H KI HOZ mice, however there do appear to be outliers in thyroid weight measurements particularly in the males. Whether this variability is related to a secondary mutation is yet to be determined. However, as the major genes responsible for PTC development have already been excluded, the mechanism of PTC formation in these mice is likely novel.

Many cancers have associated dysregulated kinase signaling whether this is through kinase overexpression, mutations in kinase genes, or defects in counter regulatory mechanisms (220, 221). As protein phosphorylation is a key regulator for numerous cellular processes, this aberrant signaling is often a driver for the cancer, rather than a consequence. Knowledge of kinase signaling in cancers is beneficial since activated kinases can be targeted with small molecule inhibitors for chemotherapy (222). While genomic changes such as a TSHR mutation in a carcinoma without any other driver mutations are the first step to understanding the mechanism of carcinogenesis, it

is essential to understand the consequences in signaling in order for this information to inform treatment and the etiology of thyroid cancer in general.

Presently, we hypothesize that MAPK signaling is responsible. While other pathways are also involved in carcinogenesis (e.g., PI3K-Akt (214)), our focus is on pathways downstream of the TSHR as the TSHR D633H mutation is clearly implicated in PTC formation. If other pathways are of relevance, phosphoproteome analysis should help to identify them. Our assessment of which pathways are important in this process is led by comparisons with the BRAF-induced PTC mouse model which no longer developed PTCs when TSHR was knocked out, and had a lower proportion of PTC without Gs (50).

The human HTC genomic results show TSHR mutations in only 4/9 samples. However, as shown in chapter 2, mutation detection of somatic mutations should be completed using an NGS methodology. It is possible the remaining samples do have TSHR mutations which are not detectable by HRM PCR due to degradation. DNA quality, as assessed by qPCR, was below threshold of CQ=35 for all samples which may have played a role in our low prevalence of TSHR mutation positive samples. Further analysis by a more sensitive methodology for a larger number of human HTCs is still necessary. Unfortunately, RNA extracted from all HTC samples was of too poor quality for further analysis as assessed by q-PCR of cDNA. Age of the blocks for the human HTCs and time between preparation of slides and analysis of slides may have played a role in this degradation.

In summary, TSHR signaling is inextricably linked to thyroid carcinoma. The role of mutations in the TSHR itself in carcinoma is supported by the development of PTC in TSHR D633H KI mice, and the occurrence of TSHR mutations in 21 published cases of thyroid carcinoma and a further three shown in the above results. This will be further explored by WES and phosphoproteome analysis of PTC samples from the TSHR D633H KI mouse model.

CHAPTER SIX: CONCLUSIONS

It can be reasonably concluded that TSHR mutations are etiologically responsible for NAH development based on the previous WES study, which found no further causative mutations (219), and the results of our tNGS study showing 96% of HTA to have TSHR or GNAS mutations in a subset of nodules with multiple samples. This knowledge validates the current molecular diagnostic methods for NAH, which include screening for TSHR and GNAS mutations.

Knowledge of the etiology of NAH is essential. In HTA, once a nodule is identified, the nodule alone (or the lobe if necessary) can be removed and molecular testing is not required. However, in the case of germline NAH (SNAH and FNAH), a total thyroidectomy is necessary to avoid development of goiter, multiple surgeries, lack of remission of hyperthyroidism during anti-thyroid drug therapy, possible heart complications or effects on a child's bone development (156, 232). Therefore, knowledge of the mutational status of a non-Graves disease hyperthyroid patient is of the utmost importance. Evaluation of DNA extracted from patient serum samples is essential for germline NAH diagnosis. Since the mutations in these cases are in all cells of the thyroid, even if there is a portion that is not showing hyperactivity at the time of a first surgery, that does not preclude this tissue from hyperactivity later in the course of the disease. Treatment by total thyroidectomy in NAH is supported by the variability of phenotype demonstrated clearly by the review of previous case reports. Different families with the same mutation can have vastly different symptoms, age of onset, and reaction to treatments. This is also true for individuals within a

family(2). Incomplete surgeries may result in follow up surgeries and hyperthyroid symptoms returning.

The importance of treatment of NAH is further emphasized by the unknown effects of TSH on other systems. This is demonstrated in the first case report, where bone effects presented before typical hyperthyroidism symptoms. TSHR signalling through alternative pathways has not been well explored. The mechanism of action of TSH on bone is not fully understood (233). Alternative G protein coupling may be responsible for TSHR signaling in bone (234).

While the TSHR is undeniably etiologically responsible for NAH, its role in carcinoma is less well explored; However, there is certainly strong evidence for its involvement. TSHR D633H KI mice developed PTC after 12 months without mutations in the major genes responsible for PTC (207). While WES is not yet complete, if the PTCs were solely due to an additional mutation, one might speculate that they: appear with a lower prevalence than 80-90%, constitute a smaller part of the thyroid, and are characterized by a higher variability regarding size from mouse to mouse. While further mutations are certainly probable based on the background of our model, the involvement of the TSHR in undeniable. Determining TSHR's true potential for induction of thyroid carcinoma in humans is essential since aberrant TSHR signaling via TSHR and GNAS mutations is found in 96% of HTA samples. The current assumption that HTA have a very low malignancy risk is based on biased retrospective data. Preliminary raw data of the first systematic review and meta-analyses for the malignancy risk of HTA indicate that the current guideline interpretation of available malignancy risk data for HTA needs to be corrected (235). Solitary HTA account for 5 - 10% of

cases of hyperthyroidism (171). It is therefore essential to determine whether TSHR mutations could be carcinoma driver mutations.

The next steps of this project will provide further specific evidence for how TSHR mutations and increased TSH signaling in general could initiate PTC. If the role of TSHR mutations can be further characterized in thyroid carcinoma, this would require a change in clinical practice for the malignancy risk assessment for HTA and for the many thyroid nodule patients. Early molecular mechanisms of thyroid cancer genesis are currently largely unknown and could serve as leads for thyroid cancer prevention. The future directions of this project may shed some light on those mechanisms and possibly elucidate the gender disparity between male and female thyroid cancer.

In summary, this thesis has identified TSHR as the sole gene responsible for NAH. The thesis has explored the phenotype of germline NAH, the consequences of late diagnosis and treatment, and the difficulties associated with delayed treatment. It has also shown a complete picture of the knowledge we have of all TSHR mediated thyroid diseases as represented in the TSHR mutation database. Finally, it has begun to explore the possibility of TSHR's role in thyroid carcinoma and proposed several future directions for this project.

7. REFERENCES

1. Stephenson A, Eszlinger M, Stewardson P, McIntyre JB, Boesenberg E, Bircan R, Sancak S, Gozu HI, Ghaznavi S, Krohn K 2020 Sensitive sequencing analysis suggests TSHR and GNAS as sole driver mutations in hot thyroid nodules. Thyroid.

2. Stephenson A, Lau L, Eszlinger M, Paschke R 2020 The Thyroid Stimulating Hormone Receptor Mutation Database Update. Thyroid.

3. Hiller-Sturmhöfel S, Bartke A 1998 The endocrine system: an overview. Alcohol health and research world **22**:153.

4. Harvey CB, Williams GR 2002 Mechanism of thyroid hormone action. Thyroid **12**:441-446.

5. Brent GA 2012 Mechanisms of thyroid hormone action. The Journal of clinical investigation **122**:3035-3043.

6. Ortiga-Carvalho TM, Sidhaye AR, Wondisford FE 2014 Thyroid hormone receptors and resistance to thyroid hormone disorders. Nature Reviews Endocrinology **10**:582.

7. Vassart G, Dumont JE 1992 The thyrotropin receptor and the regulation of thyrocyte function and growth. Endocrine reviews **13**:596-611.

8. Chiamolera MI, Wondisford FE 2009 Thyrotropin-releasing hormone and the thyroid hormone feedback mechanism. Endocrinology **150**:1091-1096.

9. Postiglione MP, Parlato R, Rodriguez-Mallon A, Rosica A, Mithbaokar P, Maresca M, Marians RC, Davies TF, Zannini MS, De Felice M, Di Lauro R 2002 Role of the thyroid-stimulating hormone receptor signaling in development and differentiation of the thyroid gland. Proc Natl Acad Sci U S A **99**:15462-15467.

10. Marians RC, Ng L, Blair HC, Unger P, Graves PN, Davies TF 2002 Defining thyrotropin-dependent and -independent steps of thyroid hormone synthesis by using thyrotropin receptor-null mice. Proc Natl Acad Sci U S A **99**:15776-15781.

11. Roger PP, Hotimsky A, Moreau C, Dumont JE 1982 Stimulation by thyrotropin, cholera toxin and dibutyryl cyclic AMP of the multiplication of differentiated thyroid cells in vitro. Mol Cell Endocrinol **26**:165-176.

12. Wynford-Thomas D, Stringer BM, Harach HR, Williams ED 1983 Control of growth in the rat thyroid--an example of specific desensitization to trophic hormone stimulation. Experientia **39**:421-423.

13. Dumont JE, Roger P, Van Heuverswyn B, Erneux C, Vassart G 1984 Control of growth and differentiation by known intracellular signal molecules in endocrine tissues: the example of the thyroid gland. Advances in cyclic nucleotide and protein phosphorylation research **17**:337-342.

14. Roger P, Taton M, Van Sande J, Dumont JE 1988 Mitogenic effects of thyrotropin and adenosine 3',5'-monophosphate in differentiated normal human thyroid cells in vitro. The Journal of clinical endocrinology and metabolism **66**:1158-1165.

15. Laugwitz KL, Allgeier A, Offermanns S, Spicher K, Van Sande J, Dumont JE, Schultz G 1996 The human thyrotropin receptor: a heptahelical receptor capable of stimulating members of all four G protein families. Proc Natl Acad Sci U S A **93**:116-120.
16. Corvilain B, Laurent E, Lecomte M, Vansande J, Dumont JE 1994 Role of the cyclic adenosine 3',5'-monophosphate and the phosphatidylinositol-Ca2+ cascades in mediating the effects of thyrotropin and iodide on hormone synthesis and secretion in human thyroid slices. The Journal of clinical endocrinology and metabolism **79**:152-159.
17. Ledent C, Dumont JE, Vassart G, Parmentier M 1992 Thyroid expression of an A2 adenosine receptor transgene induces thyroid hyperplasia and hyperthyroidism. The EMBO journal **11**:537-542.
18. Michiels FM, Caillou B, Talbot M, Dessarps-Freichey F, Maunoury MT, Schlumberger M, Mercken L, Monier R, Feunteun J 1994 Oncogenic potential of guanine nucleotide stimulatory factor alpha subunit in thyroid glands of transgenic mice. Proc Natl Acad Sci U S A **91**:10488-10492.
19. Zeiger MA, Saji M, Gusev Y, Westra WH, Takiyama Y, Dooley WC, Kohn LD, Levine MA 1997 Thyroid-specific expression of cholera toxin A1 subunit causes thyroid hyperplasia and hyperthyroidism in transgenic mice. Endocrinology **138**:3133-3140.
20. Grasberger H, Van Sande J, Hag-Dahood Mahameed A, Tenenbaum-Rakover Y, Refetoff S 2007 A familial thyrotropin (TSH) receptor mutation provides in vivo evidence that the inositol phosphates/Ca2+ cascade mediates TSH action on thyroid hormone synthesis. The Journal of clinical endocrinology and metabolism **92**:2816-2820.
21. Kero J, Ahmed K, Wettschureck N, Tunaru S, Wintermantel T, Greiner E, Schütz G, Offermanns S 2007 Thyrocyte-specific G q/G 11 deficiency impairs thyroid function and prevents goiter development. The Journal of clinical investigation **117**:2399-2407.
22. Kopp P 2012 Thyroid hormone synthesis. Werner and Ingbar's the Thyroid: A Fundamental and Clinical Text Tenth edition Lippincott Williams & Wilkins, Philadelphia:48-74.
23. Voigt C, Holzapfel H-P, Paschke R 2000 Expression of β-arrestins in toxic and cold thyroid nodules. FEBS letters **486**:208-212.
24. Frenzel R, Voigt C, Paschke R 2006 The human thyrotropin receptor is predominantly internalized by β-arrestin 2. Endocrinology **147**:3114-3122.
25. Werthmann RC, Volpe S, Lohse MJ, Calebiro D 2012 Persistent cAMP signaling by internalized TSH receptors occurs in thyroid but not in HEK293 cells. The FASEB Journal **26**:2043-2048.
26. Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL, Lefkowitz RJ 2001 Activation and targeting of extracellular signal-regulated kinases by β-arrestin scaffolds. Proceedings of the National Academy of Sciences **98**:2449-2454.
27. Ribeiro-Neto F, Urbani J, Lemee N, Lou L, Altschuler DL 2002 On the mitogenic properties of Rap1b: cAMP-induced G1/S entry requires activated and phosphorylated Rap1b. Proceedings of the National Academy of Sciences **99**:5418-5423.
28. Tsygankova OM, Saavedra A, Rebhun JF, Quilliam LA, Meinkoth JL 2001 Coordinated regulation of Rap1 and thyroid differentiation by cyclic AMP and protein kinase A. Molecular and cellular biology **21**:1921-1929.

29. Huk D, Ashtekar A, Magner A, La Perle K, Kirschner LS 2018 Deletion of Rap1b, but not Rap1a or Epac1, reduces PKA-mediated thyroid cancer. Thyroid.
30. Dumont JE, Maenhaut C, Pirson I, Baptist M, Roger PP 1991 Growth factors controlling the thyroid gland. Bailliere's clinical endocrinology and metabolism **5**:727-754.
31. Van Sande J, Parma J, Tonacchera M 1995 Somatic and germline mutations of the TSH receptor gene in thyroid diseases. Journal of Clinical Endocrinology and Metabolism **80**.
32. Dugrillon A, Bechtner G, Uedelhoven W, Weber P, Gärtner R 1990 Evidence that an iodolactone mediates the inhibitory effect of iodide on thyroid cell proliferation but not on adenosine 3', 5'-monophosphate formation. Endocrinology **127**:337-343.
33. BRENNER-GATI L, BERG KA, GERSHENGORN MC 1989 Insulin-like growth factor-I potentiates thyrotropin stimulation of adenylyl cyclase in FRTL-5 cells. Endocrinology **125**:1315-1320.
34. Roger PP, Servais P, Dumont JE 1983 Stimulation by thyrotropin and cyclic AMP of the proliferation of quiescent canine thyroid cells cultured in a defined medium containing insulin. FEBS letters **157**:323-329.
35. Smith P, Wynford-Thomas D, Stringer B, Williams E 1986 Growth factor control of rat thyroid follicular cell proliferation. Endocrinology **119**:1439-1445.
36. Gartner R 1992 Thyroid growth in vitro. Experimental and clinical endocrinology **100**:32-35.
37. Eggo MC, Bachrach LK, Burrow GN 1990 Interaction of TSH, insulin and insulin-like growth factors in regulating thyroid growth and function. Growth Factors **2**:99-109.
38. Canada TFo Accessed: June 3 2020.
39. Mircescu H. Thyroid Disease: Know the Facts. 2018).
40. Ferraz C, Paschke R 2017 Inheritable and sporadic non-autoimmune hyperthyroidism. Best Practice & Research Clinical Endocrinology & Metabolism **31**:265-275.
41. Parma J, Duprez L, Van Sande J, Hermans J, Rocmans P, Van Vliet G, Costagliola S, Rodien P, Dumont JE, Vassart G 1997 Diversity and prevalence of somatic mutations in the thyrotropin receptor and Gsα genes as a cause of toxic thyroid adenomas. The Journal of Clinical Endocrinology & Metabolism **82**:2695-2701.
42. Duprez L, Parma J, Van Sande J, Allgeier A, Leclère J, Schvartz C, Delisle M-J, Decoulx M, Orgiazzi J, Dumont J 1994 Germline mutations in the thyrotropin receptor gene cause non–autoimmune autosomal dominant hyperthyroidism. Nature genetics **7**:396.
43. Hebrant A, Van Sande J, Roger PP, Patey M, Klein M, Bournaud C, Savagner F, Leclere J, Dumont JE, van Staveren WC 2009 Thyroid gene expression in familial nonautoimmune hyperthyroidism shows common characteristics with hyperfunctioning autonomous adenomas. The Journal of Clinical Endocrinology & Metabolism **94**:2602-2609.
44. Lüblinghoff J, Nebel I, Huth S, Jäschke H, Schaarschmidt J, Eszlinger M, Paschke R 2012 The leipzig thyrotropin receptor mutation database: update 2012. European thyroid journal **1**:209-210.
45. Kleinau G, Worth CL, Kreuchwig A, Biebermann H, Marcinkowski P, Scheerer P, Krause G 2017 Structural–functional features of the thyrotropin receptor: a class A G-protein-coupled receptor at work. Frontiers in endocrinology **8**:86.

46. Agrawal N, Akbani R, Aksoy BA, Ally A, Arachchi H, Asa SL, Auman JT, Balasundaram M, Balu S, Baylin SB 2014 Integrated genomic characterization of papillary thyroid carcinoma. Cell **159**:676-690.

47. Buffet C, Hecale-Perlemoine K, Bricaire L, Dumont F, Baudry C, Tissier F, Bertherat J, Cochand-Priollet B, Raffin-Sanson M-L, Cormier F 2017 DUSP5 and DUSP6, two ERK specific phosphatases, are markers of a higher MAPK signaling activation in BRAF mutated thyroid cancers. PloS one **12**:e0184861.

48. He H, Li W, Liyanarachchi S, Jendrzejewski J, Srinivas M, Davuluri RV, Nagy R, De La Chapelle A 2015 Genetic predisposition to papillary thyroid carcinoma: involvement of FOXE1, TSHR, and a novel lincRNA gene, PTCSC2. The Journal of Clinical Endocrinology & Metabolism **100**:E164-E172.

49. Haymart MR, Repplinger DJ, Leverson GE, Elson DF, Sippel RS, Jaume JC, Chen H 2008 Higher serum thyroid stimulating hormone level in thyroid nodule patients is associated with greater risks of differentiated thyroid cancer and advanced tumor stage. The Journal of Clinical Endocrinology & Metabolism **93**:809-814.

50. Franco AT, Malaguarnera R, Refetoff S, Liao X-H, Lundsmith E, Kimura S, Pritchard C, Marais R, Davies TF, Weinstein LS 2011 Thyrotrophin receptor signaling dependence of Braf-induced thyroid tumor initiation in mice. Proceedings of the National Academy of Sciences **108**:1615-1620.

51. Iñiguez-Ariza NM, Brito JP 2018 Management of low-risk papillary thyroid cancer. Endocrinology and Metabolism **33**:185-194.

52. Lu C, Zhao L, Ying H, Willingham MC, Cheng S-y 2010 Growth activation alone is not sufficient to cause metastatic thyroid cancer in a mouse model of follicular thyroid carcinoma. Endocrinology **151**:1929-1939.

53. Paschke R, Ludgate M 1997 The thyrotropin receptor in thyroid diseases. New England Journal of Medicine **337**:1675-1681.

54. Krohn K, Maier J, Paschke R 2007 Mechanisms of disease: hydrogen peroxide, DNA damage and mutagenesis in the development of thyroid tumors. Nature Reviews Endocrinology **3**:713.

55. Parma J, Duprez L, Van Sande J, Cochaux P, Gervy C, Mockel J, Dumont J, Vassart G 1993 Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. Nature **365**:649.

56. Führer D, Holzapfel H-P, Wonerow P, Scherbaum WA, Paschke R 1997 Somatic mutations in the thyrotropin receptor gene and not in the Gsα protein gene in 31 toxic thyroid nodules. The Journal of Clinical Endocrinology & Metabolism **82**:3885-3891.

57. FÜHRER D, KUBISCH C, SCHEIBLER U, LAMESCH P, KROHN K, PASCHKE R 1998 The extracellular thyrotropin receptor domain is not a major candidate for mutations in toxic thyroid nodules. Thyroid **8**:997-1001.

58. Lyons J, Landis CA, Harsh G, Vallar L, Grunewald K, Feichtinger H, Duh Q-Y, Clark OH, Kawasaki E, Bourne HR 1990 Two G protein oncogenes in human endocrine tumors. Science **249**:655-659.

59. O'Sullivan C, Barton CM, Staddon SL, Brown CL, Lemoine NR 1991 Activating point mutations of the gsp oncogene in human thyroid adenomas. Molecular carcinogenesis **4**:345-349.
60. Paschke R, Tonacchera M, Van Sande J, Parma J, Vassart G 1994 Identification and functional characterization of two new somatic mutations causing constitutive activation of the thyrotropin receptor in hyperfunctioning autonomous adenomas of the thyroid. The Journal of Clinical Endocrinology & Metabolism **79**:1785-1789.
61. Porcellini A, Ciullo I, Laviola L, Amabile G, Fenzi G, Avvedimento V 1994 Novel mutations of thyrotropin receptor gene in thyroid hyperfunctioning adenomas. Rapid identification by fine needle aspiration biopsy. The Journal of Clinical Endocrinology & Metabolism **79**:657-661.
62. Russo D, Arturi F, Suarez HG, Schlumberger M, Du Villard J-A, Crocetti U, Filetti S 1996 Thyrotropin receptor gene alterations in thyroid hyperfunctioning adenomas. The Journal of Clinical Endocrinology & Metabolism **81**:1548-1551.
63. Russo D, Arturi F, Wicker R, Chazenbalk GD, Schlumberger M, DuVillard J, Caillou B, Monier R, Rapoport B, Filetti S 1995 Genetic alterations in thyroid hyperfunctioning adenomas. The Journal of Clinical Endocrinology & Metabolism **80**:1347-1351.
64. Vanvooren V, Uchino S, Duprez L, Costa M, Vandekerckhove J, Parma J, Vassart G, Dumont JE, Van Sande J, Noguchi S 2002 Oncogenic mutations in the thyrotropin receptor of autonomously functioning thyroid nodules in the Japanese population. European journal of endocrinology **147**:287-291.
65. Georgopoulos NA, Sykiotis GP, Sgourou A, Papachatzopoulou A, Markou KB, Kyriazopoulou V, Papavassiliou AG, Vagenakis AG 2003 Autonomously functioning thyroid nodules in a former iodine-deficient area commonly harbor gain-of-function mutations in the thyrotropin signaling pathway. European journal of endocrinology **149**:287-292.
66. Garcia-Delgado M, Gonzalez-Navarro CJ, Napal MC, Baldonado C, Vizmanos JL, Gullon A 1998 Higher sensitivity of denaturing gradient gel electrophoresis than sequencing in the detection of mutations in DNA from tumor samples. Biotechniques **24**:72, 74, 76.
67. Trulzsch B, Krohn K, Wonerow P, Paschke R 1999 DGGE is more sensitive for the detection of somatic point mutations than direct sequencing. BioTechniques **27**:266-268.
68. Trulzsch B, Krohn K, Wonerow P, Chey S, Holzapfel H-P, Ackermann F, Fuhrer D, Paschke R 2000 Detection of thyroid-stimulating hormone receptor and Gs mutations: in 75 toxic thyroid nodules by denaturing gradient gel electrophoresis. Journal of Molecular Medicine **12**:684-691.
69. Eszlinger M, Niedziela M, Typlt E, Jaeschke H, Huth S, Schaarschmidt J, Aigner T, Trejster E, Krohn K, Bösenberg E, Paschke R 2014 Somatic mutations in 33 benign and malignant hot thyroid nodules in children and adolescents. Molecular and Cellular Endocrinology **393**:39-45.
70. Calebiro D, Grassi ES, Eszlinger M, Ronchi CL, Godbole A, Bathon K, Guizzardi F, De Filippis T, Krohn K, Jaeschke H, Schwarzmayr T, Bircan R, Gozu HI, Sancak S, Niedziela M, Strom TM, Fassnacht M, Persani L, Paschke R 2016 Recurrent EZH1 mutations are a

second hit in autonomous thyroid adenomas. The Journal of Clinical Investigation **126**:3383-3388.

71. Gozu HI, Bircan R, Krohn K, Müller S, Vural S, Gezen C, Sargin H, Yavuzer D, Sargin M, Cirakoglu B, Paschke R 2006 Similar prevalence of somatic TSH receptor and Gsα mutations in toxic thyroid nodules in geographical regions with different iodine supply in Turkey. European Journal of Endocrinology **155**:535-545.

72. Sancak S, Jaeschke H, Eren F, Tarcin O, Guellueoglu B, Sen L, Sever Z, Gozu H, Bircan R, Akalin S, Paschke R, Eszlinger M 2011 High prevalence of TSHR/Gsα mutation-negative clonal hot thyroid nodules (HNs) in a Turkish cohort. Hormone and Metabolic Research **43**:562-568.

73. Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP 2011 Integrative genomics viewer. Nat Biotechnol **29**:24-26.

74. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR 2010 A method and server for predicting damaging missense mutations. Nat Methods **7**:248-249.

75. Ng PC, Henikoff S 2001 Predicting deleterious amino acid substitutions. Genome Res **11**:863-874.

76. Tonacchera M, Pinchera A 2000 Thyrotropin receptor polymorphisms and thyroid diseases. The Journal of Clinical Endocrinology & Metabolism **85**:2637-2639.

77. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP 2011 Integrative genomics viewer. Nature biotechnology **29**:24.

78. Abramowicz MJ, Duprez L, Parma J, Vassart G, Heinrichs C 1997 Familial congenital hypothyroidism due to inactivating mutation of the thyrotropin receptor causing profound hypoplasia of the thyroid gland. The Journal of clinical investigation **99**:3018-3024.

79. Khoo D, Parma J, Rajasoorya C, Ho SC, Vassart G 1999 A germline mutation of the thyrotropin receptor gene associated with thyrotoxicosis and mitral valve prolapse in a Chinese family. The Journal of Clinical Endocrinology & Metabolism **84**:1459-1462.

80. Nicoletti A, Bal M, De Marco G, Baldazzi L, Agretti P, Menabo S, Ballarini E, Cicognani A, Tonacchera M, Cassio A 2009 Thyrotropin-stimulating hormone receptor gene analysis in pediatric patients with non-autoimmune subclinical hypothyroidism. The Journal of Clinical Endocrinology & Metabolism **94**:4187-4194.

81. Cangul H, Morgan NV, Forman JR, Saglam H, Aycan Z, Yakut T, Gulten T, Tarim O, Bober E, Cesur Y 2010 Novel TSHR mutations in consanguineous families with congenital nongoitrous hypothyroidism. Clinical endocrinology **73**:671-677.

82. Arseven OK, Wilkes WP, Jameson JL, Kopp P 2000 Substitutions of tyrosine 601 in the human thyrotropin receptor result in increase or loss of basal activation of the cyclic adenosine monophosphate pathway and disrupt coupling to Gq/11. Thyroid **10**:3-10.

83. Fleming S, Thompson M, Stevens R, Heneghan C, Plüddemann A, Maconochie I, Tarassenko L, Mant D 2011 Normal ranges of heart rate and respiratory rate in children from birth to 18 years of age: a systematic review of observational studies. The Lancet **377**:1011-1018.

84. Klingmüller V, Fiedler C, Otten A 1992 Besonderheiten der Schilddrüsensonographie im Säuglings-und Kindesalter. Der Radiologe **32**:320-326.
85. Brunn J, Block U, Ruf G, Bos I, Kunze W, Scriba P 1981 Volumetric analysis of thyroid lobes by real-time ultrasound (author's transl). Deutsche medizinische Wochenschrift (1946) **106**:1338-1340.
86. Palczewska I NZ 1999 Siatki centylowe do oceny rozwoju somatycznego dzieci i mlodziezy. . Polish Institute of Mother's and Child's Health Warsaw , Poland.
87. Bossowskiego A 2013 Tyreologia wieku rozwojowego, Warsaw, Poland.
88. Zimmermann M, Hess S, Molinari L, De Benoist B, Delange F, Braverman L, Fujieda K, Ito Y, Jooste P, Moosa K 2004 New reference values for thyroid volume by ultrasound in iodine-sufficient schoolchildren: a WHO/NHD Iodine Deficiency Study Group Report. Am J Clin Nutr **79**:231-237.
89. Eszlinger M, Niedziela M, Typlt E, Jaeschke H, Huth S, Schaarschmidt J, Aigner T, Trejster E, Krohn K, Bösenberg E 2014 Somatic mutations in 33 benign and malignant hot thyroid nodules in children and adolescents. Molecular and cellular endocrinology **393**:39-45.
90. Winkler F, Kleinau G, Tarnow P, Rediger A, Grohmann L, Gaetjens I, Krause G, l 'Allemand D, Grüters A, Krude H 2010 A new phenotype of nongoitrous and nonautoimmune hyperthyroidism caused by a heterozygous thyrotropin receptor mutation in transmembrane helix 6. The Journal of Clinical Endocrinology & Metabolism **95**:3605-3610.
91. Schwab K, Gerlich M, Broecker M, Söhlemann P, Derwahl M, Lohse M 1997 Constitutively active germline mutation of the thyrotropin receptor gene as a cause of congenital hyperthyroidism. The Journal of pediatrics **131**:899-904.
92. Supornsilchai V, Sahakitrungruang T, Wongjitrat N, Wacharasindhu S, Suphapeetiporn K, Shotelersuk V 2009 Expanding clinical spectrum of non-autoimmune hyperthyroidism due to an activating germline mutation, p. M453T, in the thyrotropin receptor gene. Clinical endocrinology **70**:623-628.
93. Vaidya B, Campbell V, Tripp JH, Spyer G, Hattersley AT, Ellard S 2004 Premature birth and low birth weight associated with nonautoimmune hyperthyroidism due to an activating thyrotropin receptor gene mutation. Clinical endocrinology **60**:711-718.
94. Börgel K, Pohlenz J, Koch HG, Bramswig JH 2005 Long-term carbimazole treatment of neonatal nonautoimmune hyperthyroidism due to a new activating TSH receptor gene mutation (Ala428Val). Hormone Research in Paediatrics **64**:203-208.
95. Schwab K, Söhlemann P, Gerlich M, Broecker M, Petrykowski Wv, Holzapfel H, Paschke R, Grüters A, Derwahl M 1996 Mutations of the TSH receptor as cause of congenital hyperthyroidism. Experimental and Clinical Endocrinology & Diabetes **104**:124-128.
96. Guemas I, Guorguleva I, Rothenbuler A, Stuckens C, Weill J, Cartigny M 2003 Hyperthyroidism due to an activating mutation of the thyrotropin receptor. Horm Res **60**:98-99.
97. Alberti L, Proverbio MC, Costagliola S, Weber G, Beck-Peccoz P, Chiumello G, Persani L 2001 A novel germline mutation in the TSH receptor gene causes non-autoimmune autosomal dominant hyperthyroidism. European journal of endocrinology **145**:249-254.

98. Pohlenz J, Pfarr N, Krueger S, Hesse V 2006 Subclinical hyperthyroidism due to a thyrotropin receptor (TSHR) gene mutation (S505R). Acta paediatrica **95**:1685-1687.
99. Rodien P, Brémont C, Sanson M-LR, Parma J, Van Sande J, Costagliola S, Luton J-P, Vassart G, Duprez L 1998 Familial gestational hyperthyroidism caused by a mutant thyrotropin receptor hypersensitive to human chorionic gonadotropin. New England Journal of Medicine **339**:1823-1826.
100. Akcurin S, Turkkahraman D, Tysoe C, Ellard S, De Leener A, Vassart G, Costagliola S 2008 A family with a novel TSH receptor activating germline mutation (p. Ala485Val). European journal of pediatrics **167**:1231-1237.
101. Nishihara E, Chen C-R, Higashiyama T, Mizutori-Sasai Y, Ito M, Kubota S, Amino N, Miyauchi A, Rapoport B 2010 Subclinical nonautoimmune hyperthyroidism in a family segregates with a thyrotropin receptor mutation with weakly increased constitutive activity. Thyroid **20**:1307-1314.
102. Taha D, Adhikari A, Thirunagari R, Senguttuvan R, Flore LA 2019 MON-264 Familial Neonatal Nonautoimmune Hyperthyroidism Due To A Thyrotropin Receptor Gene Mutation (A619G). Journal of the Endocrine Society **3**:MON-264.
103. Nakamura A, Morikawa S, Aoyagi H, Ishizu K, Tajima T 2014 A Japanese family with nonautoimmune hyperthyroidism caused by a novel heterozygous thyrotropin receptor gene mutation. Pediatric research **75**:749-753.
104. Biebermann H, Schöneberg T, Krude H, Gudermann T, Grüters A 2000 Constitutively activating TSH-receptor mutations as a molecular cause of non-autoimmune hyperthyroidism in childhood. Langenbeck's archives of surgery **385**:390-392.
105. Larsen CC, Karaviti LP, Seghers V, Weiss RE, Refetoff S, Dumitrescu AM 2014 A new family with an activating mutation (G431S) in the TSH receptor gene: a phenotype discussion and review of the literature. International journal of pediatric endocrinology **2014**:23.
106. Nwosu BU, Gourgiotis L, Gershengorn MC, Neumann S 2006 Case history: a novel activating mutation in transmembrane helix 6 of the thyrotropin receptor as cause of hereditary nonautoimmune hyperthyroidism. Thyroid **16**:505-512.
107. Jaeschke H, Eszlinger M, Lueblinghoff J, Coslovsky R, Paschke R 2011 Prolonged inappropriate TSH suppression during hypothyroidism after thyroid ablation in a patient with nonautoimmune familial hyperthyroidism. Hormone and metabolic research **43**:500-504.
108. Fukata S, Hishinuma A, Nakatake N, Tajiri J 2012 A Japanese family with familial nonautoimmune hyperthyroidism with a novel mutation (Asn406Ser) in extracellular domain of thyrotrophin receptor. Clinical endocrinology (Oxford Print) **77**:329-330.
109. Okazaki Y, Arata N, Umehara N, Yamauchi T, Tajiri J, Hishinuma A, Kogai T, Idegami T, Murashima A, Sago H 2020 A CASE OF FAMILIAL NONAUTOIMMUNE HYPERTHYROIDISM DURING PREGNANCY. AACE Clinical Case Reports **6**:e94-e97.
110. Oliver-Petit I, Savagner F, Grunenwald S, Vialon M, Edouard T, Caron P 2017 Severe thyrotoxicosis in an infant revealing familial nonautoimmune hyperthyroidism with a novel (C672W) stimulating thyrotropin receptor germline mutation. Clinical case reports **5**:1980-1987.

111. Karges B, Krause G, Homoki J, Debatin K-M, de Roux N, Karges W 2005 TSH receptor mutation V509A causes familial hyperthyroidism by release of interhelical constraints between transmembrane helices TMH3 and TMH5. Journal of endocrinology **186**:377-385.
112. Fuhrer D, Paschke R 2000 Thyroid-stimulating hormone receptor mutations: update and clinical implications. Current Opinion in Endocrinology, Diabetes and Obesity **7**:288-294.
113. Biebermann H, Schöneberg T, Hess C, Germak J, Gudermann T, Grüters A 2001 The first activating TSH receptor mutation in transmembrane domain 1 identified in a family with nonautoimmune hyperthyroidism. The Journal of Clinical Endocrinology & Metabolism **86**:4429-4433.
114. Biebermann H, Krude H, Kohler B, Huhne K, Dralle H, Kohn H 1996 A novel germline mutation of the thyrotropin receptor gene leading to familial hyperthyroidism with different penetrance. J Endocrinol Invest **19**:143.
115. Lee Y-S, Poh LKS, Loke K-Y 2002 An activating mutation of the thyrotropin receptor gene in hereditary non-autoimmune hyperthyroidism. Journal of Pediatric Endocrinology and Metabolism **15**:211-216.
116. Claus M, Maier J, Paschke R, Kujat C, Stumvoll M, Führer D 2005 Novel thyrotropin receptor germline mutation (Ile568Val) in a Saxonian family with hereditary nonautoimmune hyperthyroidism. Thyroid **15**:1089-1094.
117. Liu Z, Sun Y, Dong Q, He M, Cheng CH, Fan F 2008 A novel TSHR gene mutation (Ile691Phe) in a Chinese family causing autosomal dominant non-autoimmune hyperthyroidism. Journal of human genetics **53**:475-478.
118. Arturi F, Chiefari E, Tumino S, Russo D, Squatrito S, Chazenbalk G, Persani L, Rapoport B, Filetti S 2002 Similarities and differences in the phenotype of members of an Italian family with hereditary non-autoimmune hyperthyroidism associated with an activating TSH receptor germline mutation. Journal of endocrinological investigation **25**:696-701.
119. Ferrara AM, Capalbo D, Rossi G, Capuano S, Del Prete G, Esposito V, Montesano G, Zampella E, Fenzi G, Salerno M 2007 A new case of familial nonautoimmune hyperthyroidism caused by the M463V mutation in the TSH receptor with anticipation of the disease across generations: a possible role of iodine supplementation. Thyroid **17**:677-680.
120. Elgadi A, Arvidsson C, Janson A, Marcus C, Costagliola S, Norgren S 2005 Autosomal-dominant non-autoimmune hyperthyroidism presenting with neuromuscular symptoms. Acta paediatrica **94**:1145-1148.
121. Ringkananont U, Van Durme J, Montanelli L, Ugrasbul F, Yu YM, Weiss RE, Refetoff S, Grasberger H 2006 Repulsive separation of the cytoplasmic ends of transmembrane helices 3 and 6 is linked to receptor activation in a novel thyrotropin receptor mutant (M626I). Molecular endocrinology **20**:893-903.
122. Jaeschke H, Schaarschmidt J, Eszlinger M, Huth S, Puttinger R, Rittinger O, Meiler J, Paschke R 2014 A newly discovered TSHR variant (L665F) associated with nonautoimmune hyperthyroidism in an Austrian family induces constitutive TSHR activation by steric repulsion between TM1 and TM7. The Journal of Clinical Endocrinology & Metabolism **99**:E2051-E2059.

123. Tonacchera M, Van Sande J, Cetani F, Swillens S, Schvartz C, Winiszewski P, Portmann L, Dumont JE, Vassart G, Parma J 1996 Functional characteristics of three new germline mutations of the thyrotropin receptor gene causing autosomal dominant toxic thyroid hyperplasia. The Journal of Clinical Endocrinology & Metabolism **81**:547-554.
124. Nishihara E, Nagayama Y, Amino N, Hishinuma A, Takano T, Yoshida H, Kubota S, Fukata S, KUMA K, Miyauchi A 2007 A novel thyrotropin receptor germline mutation (Asp617Tyr) causing hereditary hyperthyroidism. Endocrine journal **54**:927-934.
125. Fuhrer D, Warner J, Sequeira M, Paschke R, Gregory J, Ludgate M 2000 Novel TSHR germline mutation (Met463Val) masquerading as Graves' disease in a large Welsh kindred with hyperthyroidism. Thyroid **10**:1035-1041.
126. Führer D, Wonerow P, Willgerodt H, Paschke R 1997 Identification of a new thyrotropin receptor germline mutation (Leu629Phe) in a family with neonatal onset of autosomal dominant nonautoimmune hyperthyroidism. The Journal of Clinical Endocrinology & Metabolism **82**:4234-4238.
127. HORTON G, SCAZZIGA B, PELET B, GAUTIER E 1987 HEREDITARY HYPERTHYROIDISM WITH DIFFUSE NON-AUTOIMMUNE HYPERACTIVITY OF THE THYROID AND AUTONOMOUS FUNCTION AND GROWTH HELVETICA PAEDIATRICA ACTA. Vol 42. SCHWABE & CO AG VERLAG FARNSBURGERSTRASSE 8, CH-4132 MUTTENZ 1, SWITZERLAND, 75-75.
128. Jaeschke H, Mueller S, Eszlinger M, Paschke R 2010 Lack of in vitro constitutive activity for four previously reported TSH receptor mutations identified in patients with nonautoimmune hyperthyroidism and hot thyroid carcinomas. Clinical endocrinology **73**:815-820.
129. Alberti L, Proverbio MC, Costagliola S, Romoli R, Boldrighini B, Vigone MC, Weber G, Chiumello G, Beck-Peccoz P, Persani L 2002 Germline mutations of TSH receptor gene as cause of nonautoimmune subclinical hypothyroidism. The Journal of Clinical Endocrinology & Metabolism **87**:2549-2555.
130. Léger J, Carel JC 2018 Diagnosis and management of hyperthyroidism from prenatal life to adolescence. Best Practice & Research Clinical Endocrinology & Metabolism **32**:373-386.
131. Samuels SL, Namoc SM, Bauer AJ 2018 Neonatal thyrotoxicosis. Clinics in perinatology **45**:31-40.
132. Kopp P, Jameson JL, Roe TF 1997 Congenital nonautoimmune hyperthyroidism in a nonidentical twin caused by a sporadic germline mutation in the thyrotropin receptor gene. Thyroid **7**:765-770.
133. Kopp P, Van Sande J, Parma J, Duprez L, Gerber H, Joss E, Jameson JL, Dumont JE, Vassart G 1995 Congenital hyperthyroidism caused by a mutation in the thyrotropin-receptor gene. New England Journal of Medicine **332**:150-154.
134. Bertalan R, Sallai Á, Sólyom J, Lotz G, Szabó I, Kovács B, Szabo E, Patócs A, Rácz K 2010 Hyperthyroidism caused by a germline activating mutation of the thyrotropin receptor gene: difficulties in diagnosis and therapy. Thyroid **20**:327-332.

135. Esapa C, Duprez L, Ludgate M, Mustafa M, Kendall-Taylor P, Vassart G, Harris P 1999 A novel thyrotropin receptor mutation in an infant with severe thyrotoxicosis. Thyroid **9**:1005-1010.
136. Tonacchera M, Agretti P, Rosellini V, Ceccarini G, Perri A, Zampolli M, Longhi R, Larizza D, Pinchera A, Vitti P 2000 Sporadic nonautoimmune congenital hyperthyroidism due to a strong activating mutation of the thyrotropin receptor gene. Thyroid **10**:859-863.
137. Watkins M, Dejkhamron P, Huo J, Vazquez D, Menon R 2008 Persistent neonatal thyrotoxicosis in a neonate secondary to a rare thyroid-stimulating hormone receptor activating mutation: case report and literature review. Endocrine Practice **14**:479-483.
138. Holzapfel H-P, Wonerow P, Von Petrykowski W, Henschen M, Scherbaum W, Paschke R 1997 Sporadic congenital hyperthyroidism due to a spontaneous germline mutation in the thyrotropin receptor gene. The Journal of Clinical Endocrinology & Metabolism **82**:3879-3884.
139. Biebermann H, Winkler F, Handke D, Grüters A, Krude H, Kleinau G 2011 Molecular description of non-autoimmune hyperthyroidism at a neonate caused by a new thyrotropin receptor germline mutation. Thyroid research **4**:S8.
140. Lavard L, Sehested A, Jacobsen BB, Muller J, Perrild H, Feldt-Rasmussen U, Parma J, Vassart G 1999 Long-term follow-up of an infant with thyrotoxicosis due to germline mutation of the TSH receptor gene (Met453Thr). Hormone Research in Paediatrics **51**:43-46.
141. De Roux N, Polak M, Couet J, Leger J, Czernichow P, Milgrom E, Misrahi M 1996 A neomutation of the thyroid-stimulating hormone receptor in a severe neonatal hyperthyroidism. The Journal of Clinical Endocrinology & Metabolism **81**:2023-2026.
142. Chester J, Rotenstein D, Ringkananont U, Steuer G, Carlin B, Stewart L, Grasberger H, Refetoff S 2008 Congenital neonatal hyperthyroidism caused by germline mutations in the TSH receptor gene. Journal of Pediatric Endocrinology and Metabolism **21**:479-486.
143. Nishihara E, Fukata S, Hishinuma A, Kudo T, Ohye H, Ito M, Kubota S, Amino N, Kuma K, Miyauchi A 2006 Sporadic congenital hyperthyroidism due to a germline mutation in the thyrotropin receptor gene (Leu 512 Gln) in a Japanese patient. Endocrine journal **53**:735-740.
144. Chawla R, Alden TD, Bizhanova A, Kadakia R, Brickman W, Kopp PA 2015 Squamosal suture craniosynostosis due to hyperthyroidism caused by an activating thyrotropin receptor mutation (T632I). Thyroid **25**:1167-1172.
145. Roberts SA, Moon JE, Dauber A, Smith JR 2017 Novel germline mutation (Leu512Met) in the thyrotropin receptor gene (TSHR) leading to sporadic non-autoimmune hyperthyroidism. Journal of Pediatric Endocrinology and Metabolism **30**:343-347.
146. Wong FC, Au EY, Ip RW, Yau HC, Lit LC 2018 False-positive TSH receptor antibody results in an infant with activating TSHR mutation. Endocrine journal:EJ18-0161.
147. Kinjo S, Kanno M, Matayoshi K, Takefuta K, Izumi A, Sugisawa C, Narumi S, Kohama M 2015 A case of 3 months old Japanese boy with sporadic congenital none-autoimmune hyperthyroidism. International journal of pediatric endocrinology **2015**:P98.
148. Tomonaga K, Tahara K, Watanabe T, Ohno M, Ogawa K, Kutsukake M, Fujino A, Hishiki T, Kinjyo K, Horikawa R 2018 A case of congenital autonomous thyroid adenoma with a

somatic activating gene mutation in the thyroid-stimulating hormone receptor. Journal of Pediatric Surgery Case Reports **38**:50-52.

149. Führer D, Mix M, Wonerow P, Richter I, Willgerodt H, Paschke R 1999 Variable phenotype associated with Ser505Asn-activating thyrotropin-receptor germline mutation. Thyroid **9**:757-761.

150. Grüters A, Schöneberg T, Biebermann H, Krude H, Krohn HP, Dralle H, Gudermann T 1998 Severe congenital hyperthyroidism caused by a germ-line neo mutation in the extracellular portion of the thyrotropin receptor. The Journal of Clinical Endocrinology & Metabolism **83**:1431-1436.

151. Agretti P, De Marco G, Biagioni M, Iannilli A, Marigliano M, Pinchera A, Vitti P, Cherubini V, Tonacchera M 2012 Sporadic congenital nonautoimmune hyperthyroidism caused by P639S mutation in thyrotropin receptor gene. European journal of pediatrics **171**:1133-1137.

152. Lueblinghoff J, Mueller S, Sontheimer J, Paschke R 2010 Lack of consistent association of thyrotropin receptor mutations in vitro activity with the clinical course of patients with sporadic non-autoimmune hyperthyroidism. Journal of endocrinological investigation **33**:228-233.

153. Spambalg D, Sharifi N, Elisei R, Gross JL, Medeiros-Neto G, Fagin J 1996 Structural studies of the thyrotropin receptor and Gs alpha in human thyroid cancers: low prevalence of mutations predicts infrequent involvement in malignant transformation. The Journal of Clinical Endocrinology & Metabolism **81**:3898-3901.

154. Ludgate M, Gire V, Crisp M, Ajjan R, Weetman A, Ivan M, Wynford-Thomas D 1999 Contrasting effects of activating mutations of GαS and the thyrotropin receptor on proliferation and differentiation of thyroid follicular cells. Oncogene **18**:4798-4807.

155. Schaarschmidt J, Paschke S, Özerden M, Jäschke H, Huth S, Eszlinger M, Meller J, Paschke R 2012 Late manifestation of subclinical hyperthyroidism after goitrogenesis in an index patient with a N670S TSH receptor germline mutation masquerading as TSH receptor antibody negative Graves' disease. Hormone and Metabolic Research **44**:962-965.

156. Paschke R, Niedziela M, Vaidya B, Persani L, Rapoport B, Leclere J 2012 2012 European thyroid association guidelines for the management of familial and persistent sporadic non-autoimmune hyperthyroidism caused by thyroid-stimulating hormone receptor germline mutations. European thyroid journal **1**:142-147.

157. Mueller S, Gozu HI, Bircan R, Jaeschke H, Eszlinger M, Lueblinghoff J, Krohn K, Paschke R 2009 Cases of borderline in vitro constitutive thyrotropin receptor activity: how to decide whether a thyrotropin receptor mutation is constitutively active or not? Thyroid **19**:765-773.

158. Gozu HI, Lublinghoff J, Bircan R, Paschke R 2010 Genetics and phenomics of inherited and sporadic non-autoimmune hyperthyroidism. Molecular and cellular endocrinology **322**:125-134.

159. Kursawe R, Paschke R 2007 Modulation of TSHR signaling by posttranslational modifications. Trends in Endocrinology & Metabolism **18**:199-207.

160. Kumar S 2013 Tall stature in children: differential diagnosis and management. International journal of pediatric endocrinology **2013**:P53.
161. TAKAMATSU J, KOBE N, ITO M, OHSAWA N 1999 Body height and weight of patients with childhood onset and adult onset thyrotoxicosis. Endocrine journal **46**:S101-S103.
162. Zimmerman D, Lteif AN 1998 Thyrotoxicosis in children. Endocrinology and metabolism clinics of North America **27**:109-126.
163. Abe E, Marians RC, Yu W, Wu X-B, Ando T, Li Y, Iqbal J, Eldeiry L, Rajendren G, Blair HC 2003 TSH is a negative regulator of skeletal remodeling. Cell **115**:151-162.
164. Bassett JD, Williams AJ, Murphy E, Boyde A, Howell PG, Swinhoe R, Archanco M, Flamant F, Samarut J, Costagliola S 2008 A lack of thyroid hormones rather than excess thyrotropin causes abnormal skeletal development in hypothyroidism. Molecular endocrinology **22**:501-512.
165. Williams G 2011 Extrathyroidal expression of TSH receptor Annales d'endocrinologie. Vol 72. Elsevier, 68-73.
166. Gogakos AI, Bassett JD, Williams GR 2010 Thyroid and bone. Archives of biochemistry and biophysics **503**:129-136.
167. Laugwitz K-L, Allgeier A, Offermanns S, Spicher K, Van Sande J, Dumont JE, Schultz G 1996 The human thyrotropin receptor: a heptahelical receptor capable of stimulating members of all four G protein families. Proceedings of the National Academy of Sciences **93**:116-120.
168. Krohn K, Paschke R 2002 Somatic mutations in thyroid nodular disease. Molecular genetics and metabolism **75**:202-208.
169. Palos-Paz F, Perez-Guerra O, Cameselle-Teijeiro J, Rueda-Chimeno C, Barreiro-Morandeira F, Lado-Abeal J, Vilar DA, Argueso R, Barca O, Botana M 2008 Prevalence of mutations in TSHR, GNAS, PRKAR1A and RAS genes in a large series of toxic thyroid adenomas from Galicia, an iodine-deficient area in NW Spain. European journal of endocrinology **159**:623-631.
170. Nishihara E, Amino N, Maekawa K, Yoshida H, Ito M, Kubota S, Fukata S, Miyauchi A 2009 Prevalence of TSH receptor and Gsα mutations in 45 autonomously functioning thyroid nodules in Japan. Endocrine journal **56**:791-798.
171. Eszlinger M SA, Stewardson P, McIntyre JB, Paschke R 2018 84% Prevalence of TSHR and Gsa Mutations in 207 HTA Samples based on targeted NGS at High Depth. 88th Annual Meeting of the American Thyroid Association Washington, DC Mary Ann Liebert Inc Thyroid:

.
172. Holzapfel H-P, Führer D, Wonerow P, Weinland G, Scherbaum WA, Paschke R 1997 Identification of constitutively activating somatic thyrotropin receptor mutations in a subset of toxic multinodular goiters. The Journal of Clinical Endocrinology & Metabolism **82**:4229-4233.
173. Narumi S, Muroya K, Abe Y, Yasui M, Asakura Y, Adachi M, Hasegawa T 2009 TSHR mutations as a cause of congenital hypothyroidism in Japan: a population-based genetic epidemiology study. The Journal of Clinical Endocrinology & Metabolism **94**:1317-1323.

174. Calaciura F, Miscio G, Coco A, Leonardi D, Cisternino C, Regalbuto C, Bozzali M, Maiorana R, Ranieri A, Carta A 2002 Genetics of specific phenotypes of congenital hypothyroidism: a population-based approach. Thyroid **12**:945-951.
175. Löf C, Patyra K, Kuulasmaa T, Vangipurapu J, Undeutsch H, Jaeschke H, Pajunen T, Kero A, Krude H, Biebermann H 2016 Detection of novel gene variants associated with congenital hypothyroidism in a Finnish patient cohort. Thyroid **26**:1215-1224.
176. RUSSO D, WONG MG, COSTANTE G, CHIEFARI E, TRESELER PA, ARTURI F, FILETTI S, CLARK OH 1999 A Val 677 activating mutation of the thyrotropin receptor in a Hürthle cell thyroid carcinoma associated with thyrotoxicosis. Thyroid **9**:13-17.
177. Xu B, Reznik E, Tuttle RM, Knauf J, Fagin JA, Katabi N, Dogan S, Aleynick N, Seshan V, Middha S, Enepekides D, Casadei GP, Solaroli E, Tallini G, Ghossein R, Ganly I 2019 Outcome and molecular characteristics of non-invasive encapsulated follicular variant of papillary thyroid carcinoma with oncocytic features. Endocrine **64**:97-108.
178. Mircescu H, Parma J, Huot C, Deal C, Oligny LL, Vassart G, Van Vliet G 2000 Hyperfunctioning malignant thyroid nodule in an 11-year-old girl: pathologic and molecular studies. The Journal of pediatrics **137**:585-587.
179. Lado-Abeal J, Celestino R, Bravo S, Garcia-Rendueles M, de la Calzada J, Castro I, Castro P, Espadinha C, Palos F, Soares P 2010 Identification of a paired box gene 8–peroxisome proliferator-activated receptor gamma (PAX8–PPARγ) rearrangement mosaicism in a patient with an autonomous functioning follicular thyroid carcinoma bearing an activating mutation in the TSH receptor. Endocr Relat Cancer **17**:599-610.
180. Camacho P, Gordon D, Chiefari E, Yong S, DeJong S, Pitale S, Russo D, Filetti S 2000 A Phe 486 thyrotropin receptor mutation in an autonomously functioning follicular carcinoma that was causing hyperthyroidism. Thyroid **10**:1009-1012.
181. Bircan Rea 2005 The Second Follicular Thyroid Carcinoma Presenting as a Hot Nodule with a Somatic I486F TSH-Receptor (TSHR) Gene Mutation. Abstract Book 32nd Annual Meeting of the European Thyroid Association, 01-05092007 Leipzig.
182. Gozu H, Avsar M, Bircan R, Sahin S, Ahiskanali R, Gulluoglu B, Deyneli O, Ones T, Narin Y, Akalin S 2004 Does a Leu 512 Arg thyrotropin receptor mutation cause an autonomously functioning papillary carcinoma? Thyroid **14**:975-980.
183. Blackburn J, Giri D, Ciolka B, Gossan N, Didi M, Kokai G, Waghorn A, Jones M, Senniappan S 2018 A Rare Case of Heterozygous Gain of Function Thyrotropin Receptor Mutation Associated with Development of Thyroid Follicular Carcinoma. Case reports in genetics **2018**.
184. Niepomniszcze H, Suárez H, Pitoia F, Pignatta A, Danilowicz K, Manavela M, Elsner B, Bruno OD 2006 Follicular carcinoma presenting as autonomous functioning thyroid nodule and containing an activating mutation of the TSH receptor (T620I) and a mutation of the Ki-RAS (G12C) genes. Thyroid **16**:497-503.
185. Russo D, Arturi F, Schlumberger M, Caillou B, Monier R, Filetti S, Suarez H 1995 Activating mutations of the TSH receptor in differentiated thyroid carcinomas. Oncogene **11**:1907-1911.

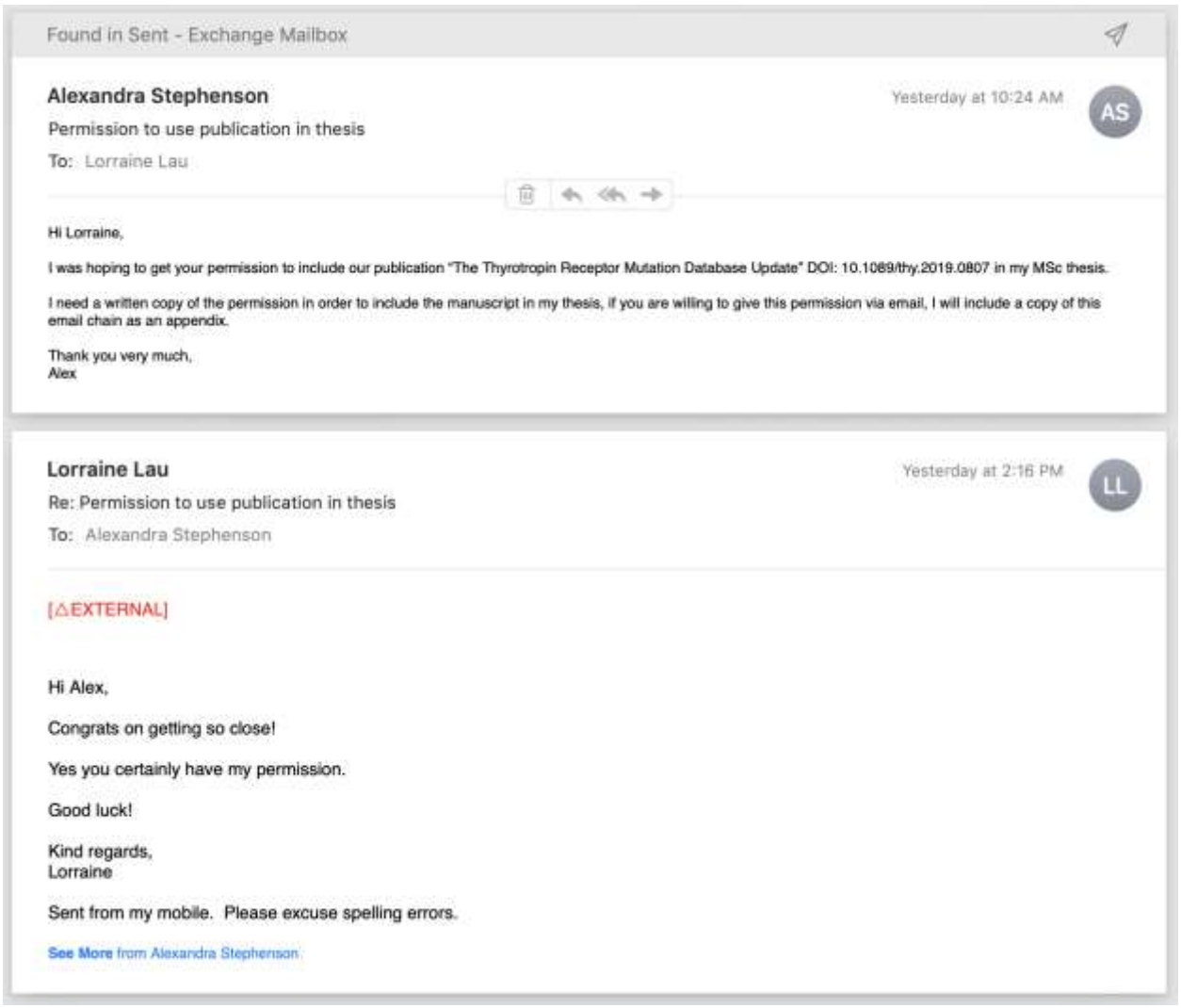
186. Führer D, Tannapfel A, Sabri O, Lamesch P, Paschke R 2003 Two somatic TSH receptor mutations in a patient with toxic metastasising follicular thyroid carcinoma and non-functional lung metastases. Endocr Relat Cancer **10**:591-600.
187. Russo D, Tumino S, Arturi F, Vigneri P, Grasso G, Pontecorvi A, Filetti S, Belfiore A 1997 Detection of an activating mutation of the thyrotropin receptor in a case of an autonomously hyperfunctioning thyroid insular carcinoma. The Journal of Clinical Endocrinology & Metabolism **82**:735-738.
188. TRÜLZSCH B, NEBEL T, PASCHKE R 1999 The thyrotropin receptor mutation database. Thyroid **9**:521-522.
189. Führer D, Lachmund P, Nebel I-T, Paschke R 2003 The thyrotropin receptor mutation database: update 2003. Thyroid **13**:1123-1126.
190. Long W, Lu G, Zhou W, Yang Y, Zhang B, Zhou H, Jiang L, Yu B 2018 Targeted next-generation sequencing of thirteen causative genes in Chinese patients with congenital hypothyroidism. Endocrine journal:EJ18-0156.
191. Zhang M-L, Sugawa H, Kosugi S, Mori T 1995 Constitutive activation of the thyrotropin receptor by deletion of a portion of the extracellular domain. Biochemical and biophysical research communications **211**:205-210.
192. Nishihara E, Chen C-R, Mizutori-Sasai Y, Ito M, Kubota S, Amino N, Miyauchi A, Rapoport B 2012 Deletion of thyrotropin receptor residue Asp403 in a hyperfunctioning thyroid nodule provides insight into the role of the ectodomain in ligand-induced receptor activation. Journal of endocrinological investigation **35**:49-53.
193. Wonerow P, Schöneberg T, Schultz G, Gudermann T, Paschke R 1998 Deletions in the Third Intracellular Loop of the Thyrotropin Receptor A NEW MECHANISM FOR CONSTITUTIVE ACTIVATION. Journal of Biological Chemistry **273**:7900-7905.
194. Qiu Y-L, Ma S-G, Liu H, Yue H-N 2016 Two novel TSHR gene mutations (p. R528C and c. 392+ 4del4) associated with congenital hypothyroidism. Endocrine research **41**:180-184.
195. Biebermann H, Schöneberg T, Krude H, Schultz Gn, Gudermann T, Grüters A 1997 Mutations of the human thyrotropin receptor gene causing thyroid hypoplasia and persistent congenital hypothyroidism. The Journal of Clinical Endocrinology & Metabolism **82**:3471-3480.
196. Camilot M, Teofoli F, Gandini A, Franceschi R, Rapa A, Corrias A, Bona G, Radetti G, Tatò L 2005 Thyrotropin receptor gene mutations and TSH resistance: variable expressivity in the heterozygotes. Clinical endocrinology **63**:146-151.
197. Calebiro D, Gelmini G, Cordella D, Bonomi M, Winkler F, Biebermann H, De Marco A, Marelli F, Libri DV, Antonica F 2012 Frequent TSH receptor genetic alterations with variable signaling impairment in a large series of children with nonautoimmune isolated hyperthyrotropinemia. The Journal of Clinical Endocrinology & Metabolism **97**:E156-E160.
198. Clifton-Bligh R, Gregory J, Ludgate M, John R, Persani L, Asteria C, Beck-Peccoz P, Chatterjee V 1997 Two novel mutations in the thyrotropin (TSH) receptor gene in a child with resistance to TSH. The Journal of Clinical Endocrinology & Metabolism **82**:1094-1100.

199. De Roux N, Misrahi M, Brauner R, Houang M, Carel J, Granier M, Le Bouc Y, Ghinea N, Boumedienne A, Toublanc J 1996 Four families with loss of function mutations of the thyrotropin receptor. The Journal of Clinical Endocrinology & Metabolism **81**:4229-4235.
200. Jordan N, Williams N, Gregory JW, Evans C, Owen M, Ludgate M 2003 The W546X mutation of the thyrotropin receptor gene: potential major contributor to thyroid dysfunction in a Caucasian population. The Journal of Clinical Endocrinology & Metabolism **88**:1002-1005.
201. Park SM, Clifton-Bligh R, Betts P, Chatterjee V 2004 Congenital hypothyroidism and apparent athyreosis with compound heterozygosity or compensated hypothyroidism with probable hemizygosity for inactivating mutations of the TSH receptor. Clinical endocrinology **60**:220-227.
202. Cangul H, Bas VN, Saglam Y, Kendall M, Barrett TG, Maher ER, Aycan Z 2014 A nonsense thyrotropin receptor gene mutation (R609X) is associated with congenital hypothyroidism and heart defects. Journal of Pediatric Endocrinology and Metabolism **27**:1101-1105.
203. Richter-Unruh A, Hauffa BP, Pfarr N, Pohlenz J 2004 Congenital primary hypothyroidism in a Turkish family caused by a homozygous nonsense mutation (R609X) in the thyrotropin receptor gene. Thyroid **14**:971-974.
204. Tiosano D, Pannain S, Vassart G, Parma J, Gershoni-Baruch R, Mandel H, Lotan R, Zaharan Y, Pery M, Weiss RE 1999 The hypothyroidism in an inbred kindred with congenital thyroid hormone and glucocorticoid deficiency is due to a mutation producing a truncated thyrotropin receptor. Thyroid **9**:887-894.
205. Mon SY, Riedlinger G, Abbott CE, Seethala R, Ohori NP, Nikiforova MN, Nikiforov YE, Hodak SP 2018 Cancer risk and clinicopathological characteristics of thyroid nodules harboring thyroid-stimulating hormone receptor gene mutations. Diagnostic cytopathology **46**:369-377.
206. Cotton R, McKusick V, Scriver C 1998 The HUGO mutation database initiative. Science **279**:10-15.
207. Jaeschke H, Undeutsch H, Patyra K, Löf C, Eszlinger M, Khalil M, Jännäri M, Makkonen K, Toppari J, Zhang F-P 2018 Hyperthyroidism and papillary thyroid carcinoma in thyrotropin receptor D633H mutant mice. Thyroid **28**:1372-1386.
208. Neumann S, Krause G, Chey S, Paschke R 2001 A free carboxylate oxygen in the side chain of position 674 in transmembrane domain 7 is necessary for TSH receptor activation. Molecular Endocrinology **15**:1294-1305.
209. Trlzsch B, Krohn K, Wonerow P, Chey S, Holzapfel H-P, Ackermann F, Fhrer D, Paschke R 2000 Detection of thyroid-stimulating hormone receptor and Gs mutations: in 75 toxic thyroid nodules by denaturing gradient gel electrophoresis. Journal of Molecular Medicine **12**.
210. Gozu HI, Bircan R, Krohn K, Müller S, Vural S, Gezen C, Sargin H, Yavuzer D, Sargin M, Cirakoglu B 2006 Similar prevalence of somatic TSH receptor and Gsα mutations in toxic thyroid nodules in geographical regions with different iodine supply in Turkey. European journal of endocrinology **155**:535-545.

211. GOZU H, AVSAR M, BIRCAN R, SAHIN S, DEYNELI O, CIRAKOGLU B, AKALIN S 2005 Mutations in the thyrotropin receptor signal transduction pathway in the hyperfunctioning thyroid nodules from multinodular goiters: a study in the Turkish population. Endocrine journal **52**:577-585.
212. Sancak S, Jaeschke H, Eren F, Tarcin O, Guellueoglu B, Sen L, Sever Z, Gozu H, Bircan R, Akalin S 2011 High prevalence of TSHR/Gsα mutation-negative clonal hot thyroid nodules (HNs) in a Turkish cohort. Hormone and metabolic research **43**:562-568.
213. 2014 Integrated genomic characterization of papillary thyroid carcinoma. Cell **159**:676-690.
214. Roger PP, van Staveren WC, Coulonval K, Dumont JE, Maenhaut C 2010 Signal transduction in the human thyrocyte and its perversion in thyroid tumors. Mol Cell Endocrinol **321**:3-19.
215. Neumann S, Krohn K, Chey S, Paschke R 2001 Mutations in the mouse TSH receptor equivalent to human constitutively activating TSH receptor mutations also cause constitutive activity. Hormone and Metabolic Research **33**:263-269.
216. Fagin JA, Mitsiades N 2008 Molecular pathology of thyroid cancer: diagnostic and clinical implications. Best practice & research Clinical endocrinology & metabolism **22**:955-969.
217. Fagin JA, Wells Jr SA 2016 Biologic and clinical perspectives on thyroid cancer. New England Journal of Medicine **375**:1054-1067.
218. Roger PP, Van Staveren WC, Coulonval K, Dumont JE, Maenhaut C 2010 Signal transduction in the human thyrocyte and its perversion in thyroid tumors. Molecular and cellular endocrinology **321**:3-19.
219. Calebiro D, Grassi ES, Eszlinger M, Ronchi CL, Godbole A, Bathon K, Guizzardi F, De Filippis T, Krohn K, Jaeschke H 2016 Recurrent EZH1 mutations are a second hit in autonomous thyroid adenomas. The Journal of clinical investigation **126**:3383-3388.
220. Hanahan D, Weinberg RA 2000 The hallmarks of cancer. cell **100**:57-70.
221. Harsha H, Pandey A 2010 Phosphoproteomics in cancer. Molecular oncology **4**:482-495.
222. Cabanillas ME, Habra MA 2016 Lenvatinib: Role in thyroid cancer and other solid tumors. Cancer treatment reviews **42**:47-55.
223. Cirulli ET, Singh A, Shianna KV, Ge D, Smith JP, Maia JM, Heinzen EL, Goedert JJ, Goldstein DB 2010 Screening the human exome: a comparison of whole genome and whole transcriptome sequencing. Genome biology **11**:R57.
224. Kassahn KS, Holmes O, Nones K, Patch A-M, Miller DK, Christ AN, Harliwong I, Bruxner TJ, Xu Q, Anderson M 2013 Somatic point mutation calling in low cellularity tumors. PloS one **8**:e74380.
225. Moran MF, Tong J, Taylor P, Ewing RM 2006 Emerging applications for phospho-proteomics in cancer molecular therapeutics. Biochimica et Biophysica Acta (BBA)-Reviews on Cancer **1766**:230-241.
226. Fu X, Fu N, Guo S, Yan Z, Xu Y, Hu H, Menzel C, Chen W, Li Y, Zeng R 2009 Estimating accuracy of RNA-Seq and microarrays with proteomics. BMC genomics **10**:161.
227. Cox J, Mann M 2007 Is proteomics the new genomics? Cell **130**:395-398.

228. Mertins P, Mani D, Ruggles KV, Gillette MA, Clauser KR, Wang P, Wang X, Qiao JW, Cao S, Petralia F 2016 Proteogenomics connects somatic mutations to signalling in breast cancer. Nature **534**:55.
229. Tong J, Taylor P, Moran MF 2014 Proteomic analysis of the epidermal growth factor receptor (EGFR) interactome and post-translational modifications associated with receptor endocytosis in response to EGF and stress. Molecular & Cellular Proteomics **13**:1644-1658.
230. Deeb SJ, D'Souza R, Cox J, Schmidt-Supprian M, Mann M 2012 Super-SILAC allows classification of diffuse large B-cell lymphoma subtypes by their protein expression profiles. Molecular & Cellular Proteomics:mcp. M111. 015362.
231. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M 2011 Andromeda: a peptide search engine integrated into the MaxQuant environment. Journal of proteome research **10**:1794-1805.
232. Ross DS, Burch HB, Cooper DS, Greenlee MC, Laurberg P, Maia AL, Rivkees SA, Samuels M, Sosa JA, Stan MN 2016 2016 American Thyroid Association guidelines for diagnosis and management of hyperthyroidism and other causes of thyrotoxicosis. Thyroid **26**:1343-1421.
233. Heemstra KA, Van der Deure WM, Peeters RP, Hamdy NA, Stokkel MP, Corssmit EP, Romijn JA, Visser TJ, Smit JW 2008 Thyroid hormone independent associations between serum TSH levels and indicators of bone turnover in cured patients with differentiated thyroid carcinoma. European journal of endocrinology **159**:69-76.
234. Büch TR, Biebermann H, Kalwa H, Pinkenburg O, Hager D, Barth H, Aktories K, Breit A, Gudermann T 2008 G13-dependent activation of MAPK by thyrotropin. Journal of Biological Chemistry **283**:20330-20341.
235. Lau L FA, Robertson H, Stephenson A, Rabi DM, Paschke R 2019 Malignancy Risk of Hyperfunctioning Thyroid Nodules Compared with Non-Toxic Thyroid Nodules: Systematic Review and Meta Analysis 89th Annual Meeting of the American Thyroid Association. Thyroid **29**:P-1-A-166.
236. Lee ES, Kim J-h, Na DG, Paeng JC, Min HS, Choi SH, Sohn CH, Chang K-H 2013 Hyperfunction thyroid nodules: their risk for becoming or being associated with thyroid cancers. Korean journal of radiology **14**:643-652.
237. Gabalec F, Svilias I, Plasilova I, Hovorkova E, Ryska A, Horacek J 2014 Follicular variant of papillary carcinoma presenting as a hyperfunctioning thyroid nodule. Journal of pediatric hematology/oncology **36**:e94-e96.

Appendix A: Copyright and Letter of Permission from all co-authors



Found in Sent - Exchange Mailbox

Alexandra Stephenson

Yesterday at 10:24 AM

AS

Permission to use publication in thesis

To: Lorraine Lau

Hi Lorraine,

I was hoping to get your permission to include our publication "The Thyrotropin Receptor Mutation Database Update" DOI: 10.1089/thy.2019.0807 in my MSc thesis.

I need a written copy of the permission in order to include the manuscript in my thesis, if you are willing to give this permission via email, I will include a copy of this email chain as an appendix.

Thank you very much,
Alex

Lorraine Lau

Yesterday at 2:16 PM

LL

Re: Permission to use publication in thesis

To: Alexandra Stephenson

[△EXTERNAL]

Hi Alex,

Congrats on getting so close!

Yes you certainly have my permission.

Good luck!

Kind regards,
Lorraine

Sent from my mobile. Please excuse spelling errors.

See More from Alexandra Stephenson

Good morning,

I am writing because I am a graduate student with two first author papers in Thyroid. I am graduating from my MSc this summer and will be preparing a manuscript based thesis. I would like to include these two papers in their entirety. The purpose of request is to include these your thesis which will be added to the institutional repository at the University of Calgary and the Library and Archives Canada. Here are the links to access further information:

University of Calgary Theses Repository – The Vault http://theses.ucalgary.ca/

Library and Archives Canada http://collectionscanada.gc.ca/obj/s4/f2/frm-nl59-2-e.pdf

Here are the citations I would like to include:
https://doi.org/10.1089/thy.2019.0648
https://doi.org/10.1089/thy.2019.0807

Thank you very much for your time, I look forward to your reply.

Best regards,

Alex Stephenson

Ballen, Karen Yesterday at 3:30 PM

RE: Inclusion of manuscripts in thesis

To: Alexandra Stephenson

[△EXTERNAL]

Dear Alex:

Copyright permission is granted to include preprints of these articles, but because Open Access was not ordered, there is a one-yr embargo from the date of publication in print before you may post the published version.

Kind regards,

Karen Ballen
Manager, Liebert Open Access, Reprints and ePrints

Bösenberg, Eileen 2:49 AM

AW: Manuscript permission

To: Alexandra Stephenson

[△EXTERNAL]

Hi Alex,

I am happy to give you my permission to include our publication "Sensitive Sequencing Analysis Suggests Thyrotropin Receptor and Guanine Nucleotide-Binding Protein G Subunit Alpha as Sole Driver Mutations in Hot Thyroid Nodules" DOI: 10.1089/thy.2019.0648 in your MSc thesis.

Good luck and all the best,
Eileen

Von: Alexandra Stephenson <alexandra.stephen1@ucalgary.ca>
Gesendet: Donnerstag, 2. Juli 2020 18:37
An: Bösenberg, Eileen <Eileen.Boesenberg@medizin.uni-leipzig.de>
Betreff: Manuscript permission

Krohn, Knut 2:20 AM

AW: Manuscript permission

To: Alexandra Stephenson, krok@medizin.uni-leipzig.de

[△EXTERNAL]

Dear Alex,
no problem. I herewith grant permission to include our publication "Sensitive Sequencing Analysis Suggests Thyrotropin Receptor and Guanine Nucleotide-Binding Protein G Subunit Alpha as Sole Driver Mutations in Hot Thyroid Nodules" DOI: 10.1089/thy.2019.0648 in your MSc thesis.
Take care,
Knut

PD Dr. Knut Krohn
Leiter der Core Unit DNA Technologien
Core Unit DNA, Liebigstraße 19/21, 04103 Leipzig
Fon: 0341-9715980
Fax: 0341-9715979
Universität Leipzig
Ritterstraße 26, 04109 Leipzig
Körperschaft des Öffentlichen Rechts.
Vertreten durch die Rektorin Prof. Dr. med. Beate A. Schücking
Zuständige Aufsichtsbehörde: Sächsisches Staatsministerium für Wissenschaft und Kunst,
Wigardstraße 17, 01097 Dresden, www.smwk.de
Umsatzsteuer-Identifikationsnummer gemäß § 27 a Umsatzsteuergesetz: DE 141510383

Von: Alexandra Stephenson <alexandra.stephen1@ucalgary.ca>
Gesendet: Donnerstag, 2. Juli 2020 18:35:07
An: krok@medizin.uni-leipzig.de
Betreff: Manuscript permission

☆ **rifat bircan** 8:38 AM

Re: Manuscript permission

To: Alexandra Stephenson

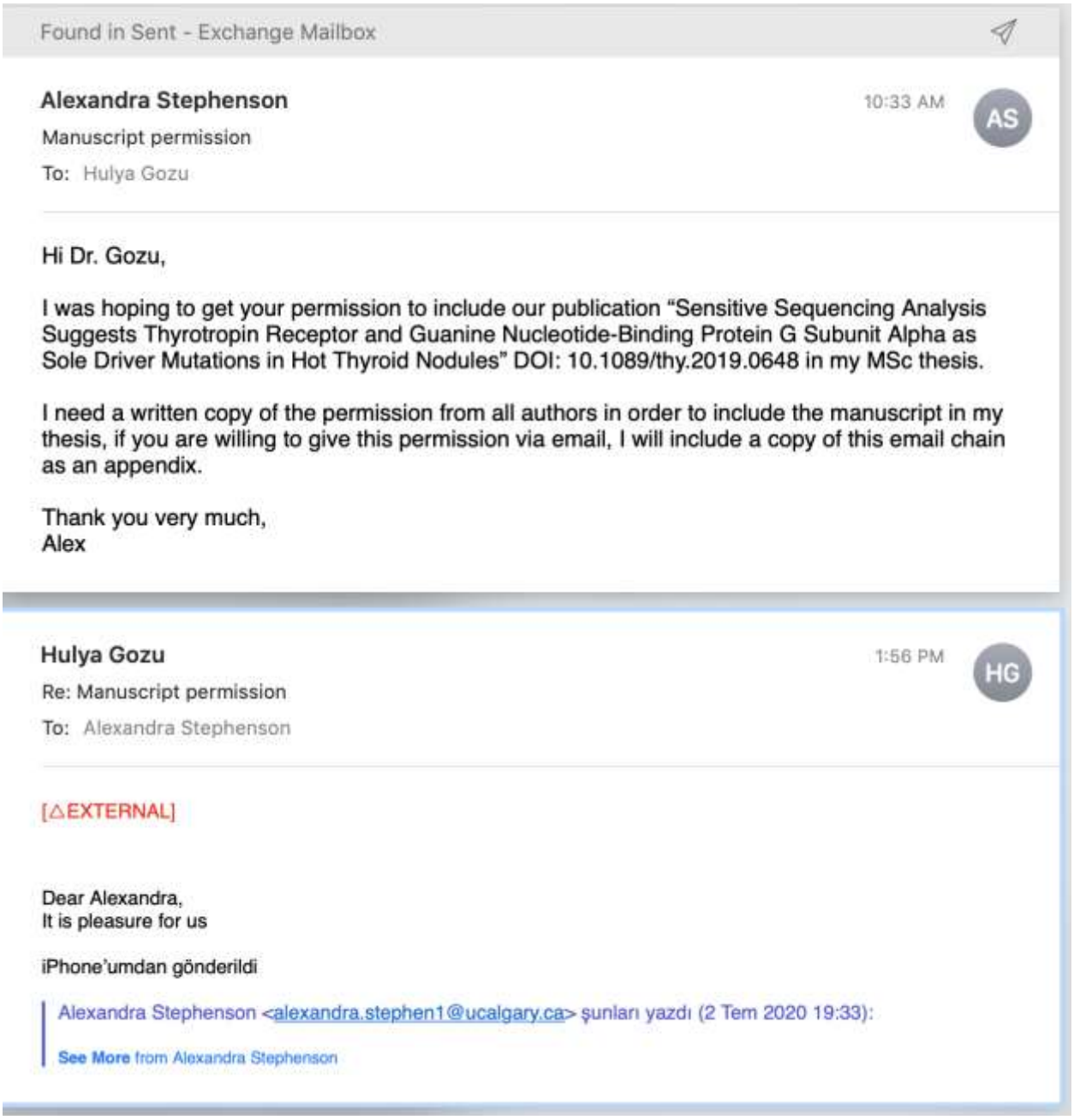
[△EXTERNAL]

Dear Alexandra;

I grant permission for you to use the publication ""Sensitive Sequencing Analysis Suggests Thyrotropin Receptor and Guanine Nucleotide-Binding Protein G Subunit Alpha as Sole Driver Mutations in Hot Thyroid Nodules" DOI: 10.1089/thy.2019.0648".

Best wishes.

Dr. Rifat Bircan



Found in Sent - Exchange Mailbox

Alexandra Stephenson 10:33 AM

Manuscript permission

To: Hulya Gozu

Hi Dr. Gozu,

I was hoping to get your permission to include our publication "Sensitive Sequencing Analysis Suggests Thyrotropin Receptor and Guanine Nucleotide-Binding Protein G Subunit Alpha as Sole Driver Mutations in Hot Thyroid Nodules" DOI: 10.1089/thy.2019.0648 in my MSc thesis.

I need a written copy of the permission from all authors in order to include the manuscript in my thesis, if you are willing to give this permission via email, I will include a copy of this email chain as an appendix.

Thank you very much,
Alex

Hulya Gozu 1:56 PM

Re: Manuscript permission

To: Alexandra Stephenson

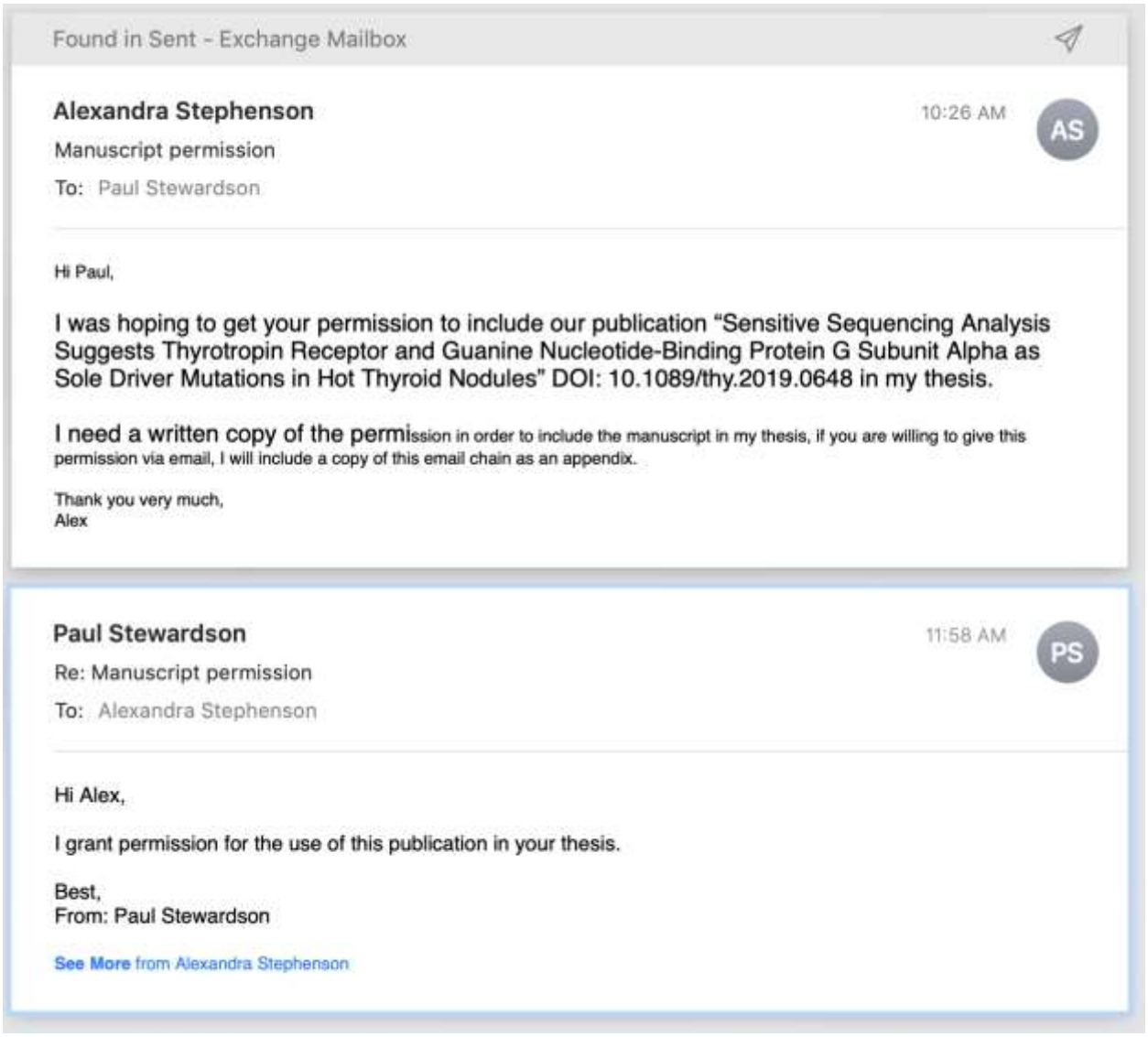
[△EXTERNAL]

Dear Alexandra,
It is pleasure for us

iPhone'umdan gönderildi

Alexandra Stephenson <alexandra.stephen1@ucalgary.ca> şunları yazdı (2 Tem 2020 19:33):

See More from Alexandra Stephenson



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Alexandra Stephenson 10:26 AM

Manuscript permission

To: Paul Stewardson

Hi Paul,

I was hoping to get your permission to include our publication "Sensitive Sequencing Analysis Suggests Thyrotropin Receptor and Guanine Nucleotide-Binding Protein G Subunit Alpha as Sole Driver Mutations in Hot Thyroid Nodules" DOI: 10.1089/thy.2019.0648 in my thesis.

I need a written copy of the permission in order to include the manuscript in my thesis, if you are willing to give this permission via email, I will include a copy of this email chain as an appendix.

Thank you very much,
Alex

Paul Stewardson 11:58 AM

Re: Manuscript permission

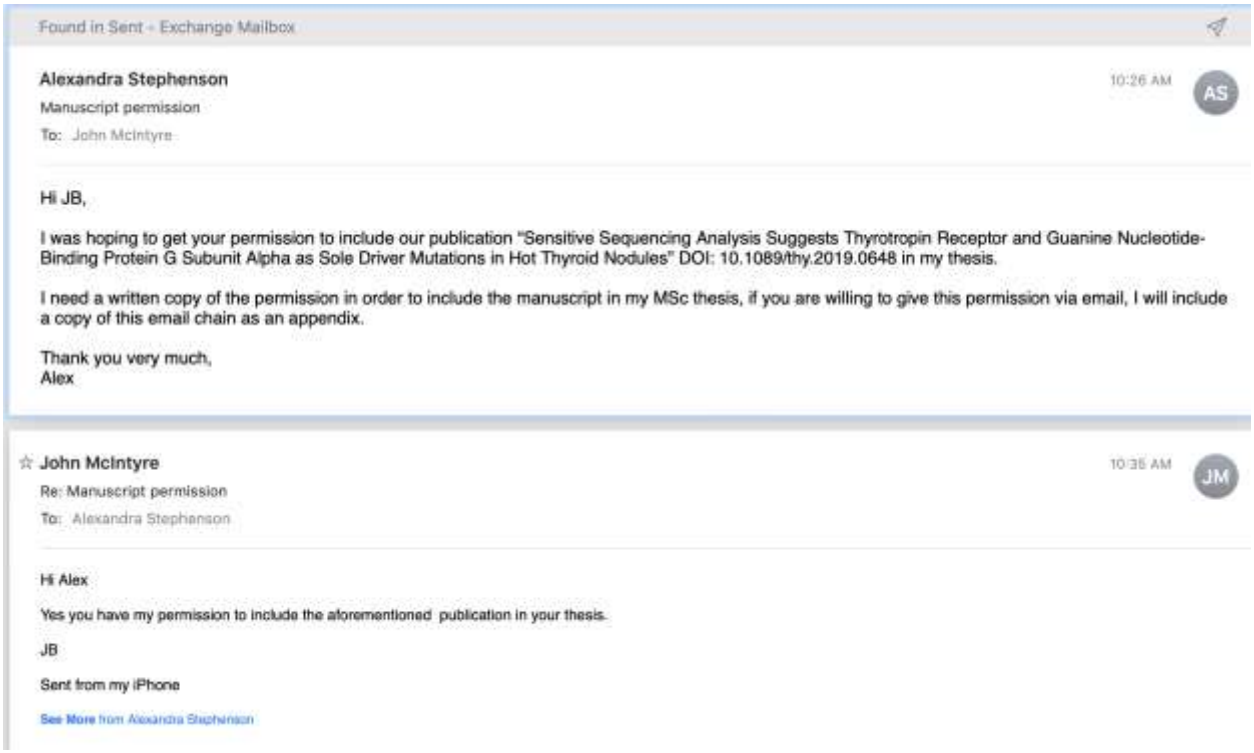
To: Alexandra Stephenson

Hi Alex,

I grant permission for the use of this publication in your thesis.

Best,
From: Paul Stewardson

See More from Alexandra Stephenson



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Alexandra Stephenson 10:26 AM

Manuscript permission

To: John McIntyre

Hi JB,

I was hoping to get your permission to include our publication "Sensitive Sequencing Analysis Suggests Thyrotropin Receptor and Guanine Nucleotide-Binding Protein G Subunit Alpha as Sole Driver Mutations in Hot Thyroid Nodules" DOI: 10.1089/thy.2019.0648 in my thesis.

I need a written copy of the permission in order to include the manuscript in my MSc thesis, if you are willing to give this permission via email, I will include a copy of this email chain as an appendix.

Thank you very much,
Alex

☆ **John McIntyre** 10:35 AM

Re: Manuscript permission

To: Alexandra Stephenson

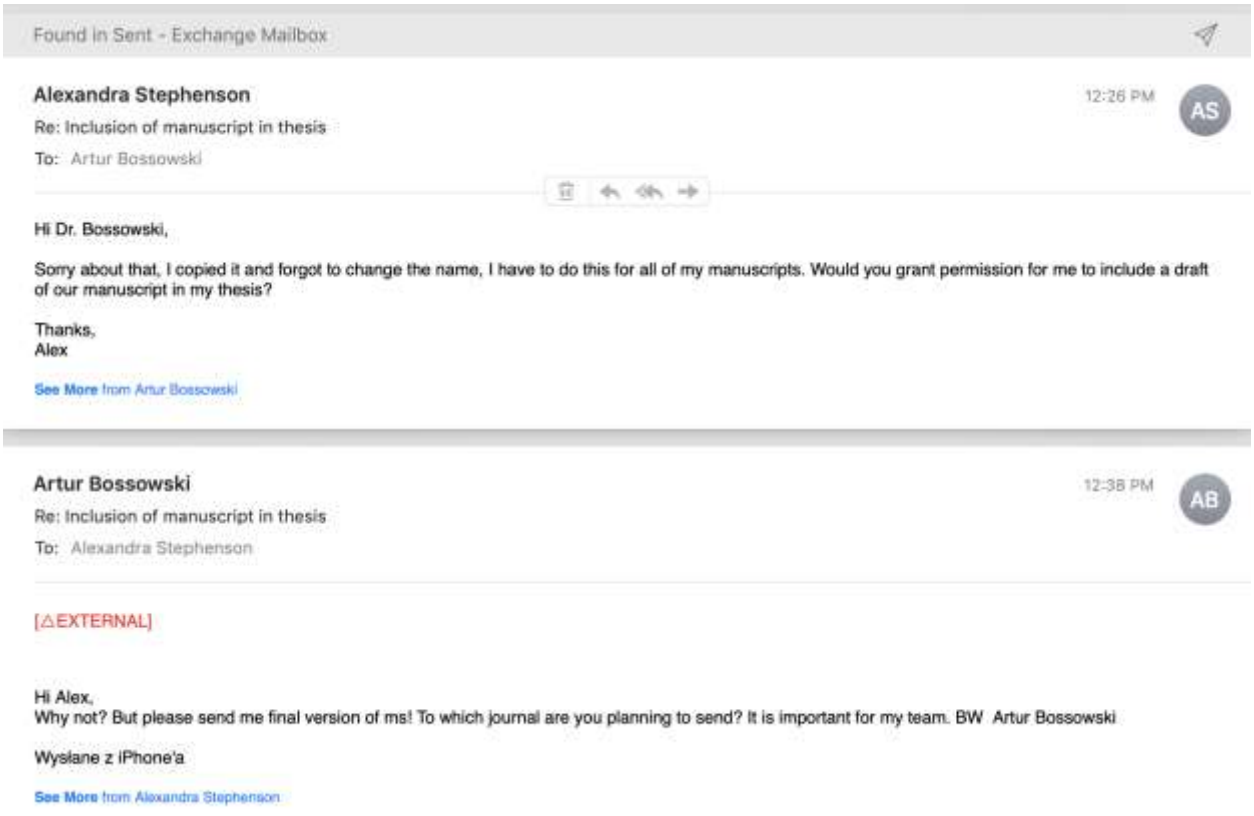
Hi Alex

Yes you have my permission to include the aforementioned publication in your thesis.

JB

Sent from my iPhone

See More from Alexandra Stephenson



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Alexandra Stephenson 12:26 PM AS

Re: Inclusion of manuscript in thesis

To: Artur Bossowski

Hi Dr. Bossowski,

Sorry about that, I copied it and forgot to change the name, I have to do this for all of my manuscripts. Would you grant permission for me to include a draft of our manuscript in my thesis?

Thanks,
Alex

See More from Artur Bossowski

Artur Bossowski 12:38 PM AB

Re: Inclusion of manuscript in thesis

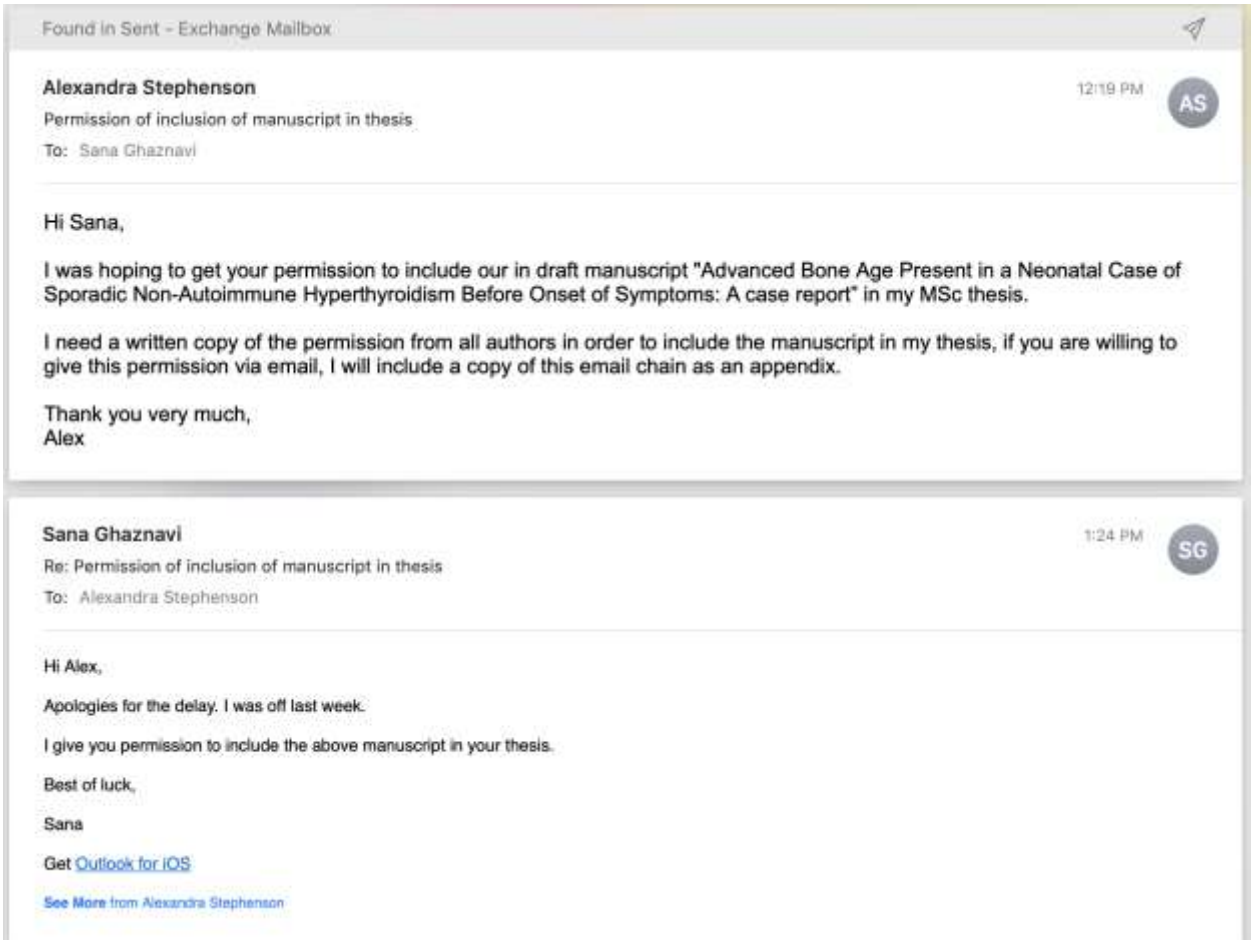
To: Alexandra Stephenson

[△EXTERNAL]

Hi Alex,
Why not? But please send me final version of ms! To which journal are you planning to send? It is important for my team. BW Artur Bossowski

Wysłane z iPhone'a

See More from Alexandra Stephenson



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Alexandra Stephenson 12:19 PM

Permission of inclusion of manuscript in thesis

To: Sana Ghaznavi

Hi Sana,

I was hoping to get your permission to include our in draft manuscript "Advanced Bone Age Present in a Neonatal Case of Sporadic Non-Autoimmune Hyperthyroidism Before Onset of Symptoms: A case report" in my MSc thesis.

I need a written copy of the permission from all authors in order to include the manuscript in my thesis, if you are willing to give this permission via email, I will include a copy of this email chain as an appendix.

Thank you very much,
Alex

Sana Ghaznavi 1:24 PM

Re: Permission of inclusion of manuscript in thesis

To: Alexandra Stephenson

Hi Alex,

Apologies for the delay. I was off last week.

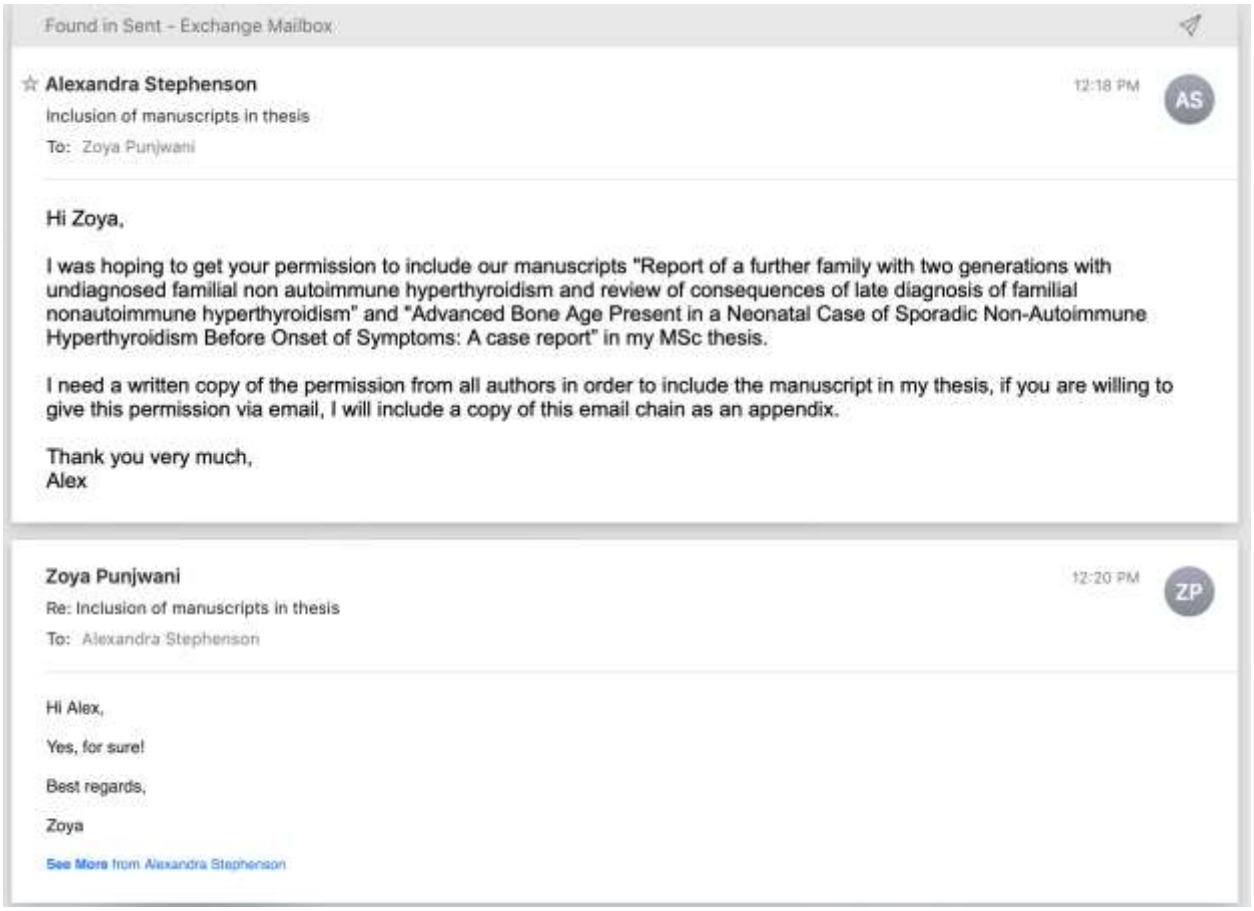
I give you permission to include the above manuscript in your thesis.

Best of luck,

Sana

Get Outlook for iOS

See More from Alexandra Stephenson



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☆ **Alexandra Stephenson** 12:18 PM

Inclusion of manuscripts in thesis

To: Zoya Punjwani

Hi Zoya,

I was hoping to get your permission to include our manuscripts "Report of a further family with two generations with undiagnosed familial non autoimmune hyperthyroidism and review of consequences of late diagnosis of familial nonautoimmune hyperthyroidism" and "Advanced Bone Age Present in a Neonatal Case of Sporadic Non-Autoimmune Hyperthyroidism Before Onset of Symptoms: A case report" in my MSc thesis.

I need a written copy of the permission from all authors in order to include the manuscript in my thesis, if you are willing to give this permission via email, I will include a copy of this email chain as an appendix.

Thank you very much,
Alex

Zoya Punjwani 12:20 PM

Re: Inclusion of manuscripts in thesis

To: Alexandra Stephenson

Hi Alex,

Yes, for sure!

Best regards,

Zoya

See More from Alexandra Stephenson

Appendix B: Pathologist's notes and summary of publication for human HTC samples

Sample 1 (S06-29629)was sent to us by Dr. Jihn Kim and was previously published(236). This sample came from a 47-year-old male with 2 conventional PTCs and one hyperfunctioning PTC with a TSH<0.05. The recorded size of the hyperfunctioning PTC was 1.1 cm; no molecular analysis had been completed. Dr. Khalil thought the sample likely came from a patient with Graves Disease. Most tissue is fibrous and would therefore be cold on the scan, thus is not a hyperfunctioning PTC. Dr. Khalil diagnosed this sample as an invasive micro-follicular variant PTC based on the following criteria: it has no boundary but nuclear features such as round and granular nuclei which are enlarged, irregularly contoured, overlapping and crowded with fine, powdery gradient are present. Surrounding hyperplastic tissue has tall columnar cells, similar to papillary structure however non-neoplastic. Dr. Khalil measured the size to be 0.6 cm. Dr. Khalil suggested that this is a cold nodule, likely surrounded by hot tissue due to Graves disease. fT3, fT4, TPO antibodies and TSHR antibodies were requested from publishing author but were not received.

Sample 2 (S05-25910) was sent to us by Dr. Jihn Kim and was previously published(236). This sample came from a 63-year-old female with 1 euthyroid PTC and 1 hot FTC with TSH<0.02. The recorded size of the hyperfunctioning FTC was 2.6 cm; no molecular analysis had been completed. Dr. Khalil's observations were that this is a 0.9 cm microcarcinoma with a ring of lymphoid tissue separating fat cells from carcinoma. He thought it is likely that the lymphoid tissue was diluting DNA for molecular analysis. Scintigraphy, fT3 and fT4 measurements were requested from the publishing author but were not received.

Sample 3 (07B543, normal is 06B17562N) was sent to us by Dr. Jose Manuel Camselle and Dr. Lado-Abeal and was previously published(179). It came from a 55-year-old female with hyperfunctioning FTC. Thyroid hormone values were as follows: TSH <0.01, fT4 was 1.62 ng/dl, 20.9 pmol/l (normal range 0.85–1.69 ng/dl), and fT3 was 6.25 pg/ml, 96.25 nmol/l (normal range 2.30–4.30 pg/ml). Previous molecular analysis showed TSHR M453T and PAX8/PPARG. Dr. Khalil observed a papillary thyroid carcinoma with incomplete surrounding fibrous band and vascular invasion. The subtype was difficult to classify, as the tumour has a thick colloid which

would indicate fvPTC but pink chromatin is specific to solid PTC. Tumour has optically clear nuclei with visible grooves and a membrane attached nucleolous with accentuation of nuclear membrane. Scintigraphy was requested from the publishing author but was not available.

Sample 4 (B1183) was sent to us by Dr. Filip Gabalec and was previously published(237). It came from a 15-year-old female with fvPTC. Thyroid hormone levels were as follows: TSH<0.01, free thyroxine (fT4) concentration was elevated to 30.6 pmol/L (reference range, 7 to 15.9 pmol/L). No molecular analysis had been completed. Dr. Khalil observed capsular invasion, however it may be a site of an FNA which would make PTC unlikely, possibly a NIFTP. Follicular architecture with many small follicles. The researcher who sent us this sample confirmed that this patient had an FNA but could not confirm the location.

Sample 5 (1866-95) is the first of 4 unpublished Greek samples for which we do not have any patient information. Efforts have been made to get information with Dr. Pazaitou). Unpublished mutational analysis in Germany showed TSHR D727E, BRAF and NRAS mutations. Dr. Khalil observed a microPTC, 0.7 cm and either hyperplastic nodule, additional carcinoma or follicular adenoma adjacent to tumour. The nuclear features were not sufficient to classify this as carcinoma; however he noted that this could be the hot tissue, and rather than the PTC which has significant fibrous tissue. These two areas were extracted separately as per Dr. Khalil's recommendation.

Sample 6 (599-99) is the second unpublished Greek sample. Mutational analysis in Germany showed TSHR D727E, TSHR D617D, and BRAF mutations. Dr. Khalil noted that it was an invasive tumour with thick colloid, multinucleated cells and distinct papillae. These features indicate PTC.

Sample 7 (6148-03) is the third unpublished Greek sample. Mutational analysis in Germany showed TSHR N674D and BRAF mutations. Dr. Khalil observed papillary architecture with invasiveness. He also noted large nuclei and papillae which are indicative of PTC.

Sample 8 (1389-9) is the fourth unpublished Greek sample. Mutational analysis in Germany showed TSHR D727E and C672T mutations. Dr. Khalil noted closely packed tumour follicles reaching beyond the fibrous band making it invasive. He also remarked on the follicular architecture and noted that the nuclear features are not precise for PTC. While he was certain this tumour was a well differentiated thyroid carcinoma, it was challenging to categorize it further however his decision was to diagnose PTC based on multinucleate cells, enlarged and round nuclei, and colloid features that align with PTC.

Sample 9 was sent to us by Dr. Guy Van-Vliet and was previously published(178). This sample came from a 11 year old female with PTC and a TSH measurement of 0.03. Unfortunately for this sample we only received previously extracted DNA and a block preserved in Bouin solution so no further pathological evaluations are available.