MOLECULAR AND CLINICAL IMPLICATIONS OF THYROID HORMONE TRANSPORT AND ACTION

ANNA LIYA MARIA VAN GUCHT

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The printing of this thesis has been financially supported by the Erasmus University Rotterdam, and further financial support was kindly provided by:
Schildklier Organisatie Nederland
Ipsen Pharmaceuticals
Goodlife Healthcare
Ferring
Chipsoft
Pfizer
Eisai

ISBN: 978-94-6299-656-4

Cover design by: Emma Anquinet
Layout: Ridderprint BV - www.ridderprint.nl
Printed by: Ridderprint BV - www.ridderprint.nl

Molecular and Clinical Implications of Thyroid Hormone Transport and Action

Moleculaire en klinische implicaties van schildklierhormoontransport en -actie

Proefschrift

Ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
dinsdag 10 oktober 2017 om 15.30 uur

door

Anna Liya Maria van Gucht

geboren te Moskou, Rusland

Erasmus University Rotterdam

Erasmus

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GENERAL INTRODUCTION

1

Introduction

Thyroid hormone (TH) is essential for normal development and cellular metabolism. Because of the importance of TH for the developing brain, hypothyroidism - a deficiency of TH - during early development can lead to severe defects in motor and mental development as illustrated by patients with untreated congenital hypothyroidism.[1,2] In adults, hypothyroidism can result in symptoms such as cold intolerance, fatigue, constipation and depression. In contrast, an excess of thyroid hormone -hyperthyroidism - can cause an upregulation of metabolism leading to heat intolerance, weight loss and tachycardia.[3,4] Serum TH levels are regulated by the hypothalamic-pituitary-thyroid (HPT) axis. The hypothalamus produces thyrothropin releasing hormone (TRH), which stimulates the production of thyroid-stimulating hormone (TSH) by the pituitary. TSH stimulates the thyroid gland to produce TH, predominantly the prohormone T4 and a small fraction of the bioactive hormone T3.[5,6] In turn, T3 and T4 negatively regulate TRH and TSH synthesis as part of a feedback control mechanism. TH action is regulated at the cellular level: i.e. the intracellular TH availability is dependent on membrane transport and TH metabolism is mediated by deiodinating enzymes in the cell. The importance of these regulatory steps is illustrated by the severe consequences in patients with defects in thyroid hormone transport or deiodination (see below). Ultimately, T3 binds to TH receptors (TRs) in the nucleus, thereby modulating gene expression of T3-target genes.[7]

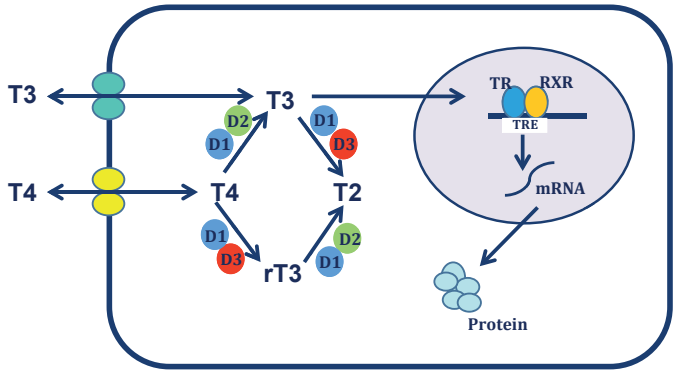

Figure 1. TH transport, metabolism and action in a T3 target cell (adapted from Visser T.J. et al, Horm Res 2007)

Thyroid hormone synthesis

TH is produced by the thyroid gland, which is composed of follicles, constituted of thyrocytes that surround the colloid lumen. Iodide is required for TH synthesis. Therefore the Na/I symporter (NIS; SLC5A5) transports iodide across the basolateral membrane of the thyrocyte, after which the transporter Pendrin (SLC26A4) facilitates the efflux of iodide to the colloid lumen. In the colloid, iodide is oxidized by thyroperoxidase (TPO), which subsequently incorporates the oxidized iodide into tyrosyl residues of thyroglobulin (TG). By this organification reaction, mono- and diiodotyrosines (MIT and DIT) are formed. TPO also catalyzes the coupling of two DIT residues to form T4 and the addition of a MIT to a DIT, which results in T3. After proteolysis of thyroglobulin in lysosomes T4 and T3 are secreted into the circulation by transporters, including the monocarboxylate transporter MCT8. Iodine is recycled by deiodination of iodothyronines by iodotyrosine dehalogenase (DEHAL1).[8–11]

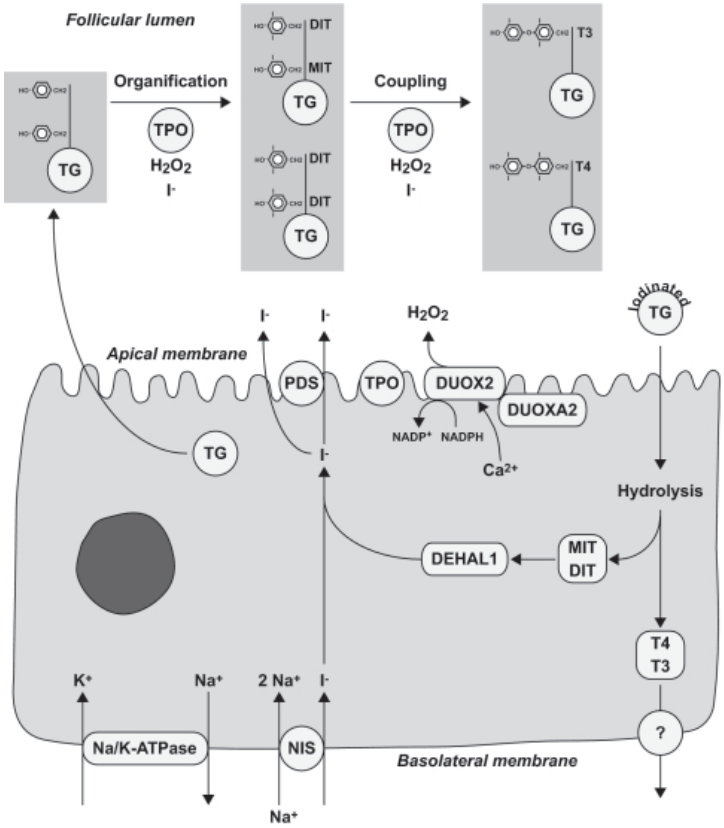

Figure 2. Main steps in TH synthesis. By A. Bizhanova and P. Kopp, Endocrinology 2009

Thyroid hormone transport

Since TH metabolism and action take place intracellular, TH transport across the plasma membrane is required. Initially, it was believed that the transfer of TH across the cell membrane occurred via passive diffusion. This assumption was based on the fact that TH has a lipophilic structure and thus could easily cross the lipid bilayer of the plasma membrane. Then a number of studies showed that TH uptake was saturable, ATP- and Na^+-dependent in certain cell types, providing evidence for transporter proteins being responsible for TH translocation over the cell membrane.[12,13] During the last decade, several TH transporter families in different species have been identified, including monocarboxylate transporters (MCTs), organic anion transporter polypeptides (OATPs), L-type amino acid transporters (LATs) and Na^+/taurocholate co-transporting polypeptides (NTCP).[14–22]

MCT8

MCT8 (SLC16A2) belongs to the MCT family of transporters, comprising 14 members. Because MCT1–4 showed transport of monocarboxylates such as lactate and pyruvate, the family was named after this class of substrates.[16,23] MCTs are proteins with 12 predicted transmembrane domains with intracellularly localized N- and C-terminal domains. In humans, the *MCT8* gene is located on the X-chromosome (Xq13.2) and contains 6 exons. First, the rat Mct8 (rMct8) was identified as a specific thyroid hormone transporter,[14] after which the human MCT8 (hMCT8) was functionally characterized.[14,24] Several studies confirmed that the expression of hMCT8 markedly increased intracellular T3 and T4, and modestly increased concentrations of the TH metabolites rT3 and 3,3'-T2. After 10–30 minutes, TH uptake by hMCT8 reached a plateau phase, suggesting that MCT8 facilitates both TH uptake and efflux. This is supported by the observation that co-transfection of μ-crystallin (CRYM), a high-affinity TH-binding protein in cytoplasm, showed a decrease in TH efflux and an increase in TH uptake by hMCT8.[25] MCT8/ Mct8 is expressed in liver, kidney, thyroid, heart, brain, and placenta during gestation.[26–29] Mutations in MCT8 are associated with the Allan-Herndon-Dudley syndrome (AHDS), an X-linked disorder leading to severe psychomotor retardation.[30,31] The clinical phenotype of patients with AHDS comprises severe mental retardation, axial hypotonia, dystonia, defective speech development, low body weight and muscular hypoplasia. On brain MRI a delayed myelination is seen and thyroid function testing shows increased serum T3, low T4 levels and a normal to elevated TSH.[32–34] The pathogenesis of AHDS is not yet completely understood, but it is hypothesized that inactivating mutations in MCT8 result in a hypothyroid state of MCT8-expressing neurons.[35–38] Different *Mct8* knock-

out (KO) mouse models have been studied to explore the molecular mechanisms underlying the neurological and endocrinological abnormalities in ADHS patients.[26,39] *Mct8* KO mice have similar endocrine abnormalities as ADHS patients. However, *Mct8* KO mice lack an overt neurological phenotype despite low levels of T3 and T4 in the brain.[26,38,39] Several explanations for this have been postulated. First, the consequences of hypothyroidism on brain development in mice may differ from humans. Second, hMCT8 may be important for the transport of other ligands, essential for normal brain development in humans. Third, *Mct8* deficiency may sufficiently be compensated by other TH transporters in mouse brain, such as Oatp1c1, Lat1 or Lat2. *Oatp1c1* KO mice indeed show decreased T3 and T4 levels in the brain together with downregulation of TH responsive genes.[38,40–45] *Mct8/Oatp1c1* double KO (dKO) mice have a significant neurological phenotype together with a clear hypothyroid state in brain.[41]

MCT10

Another member of the MCT family is MCT10 (SLC16A10), also known as a T-type amino acid transporter (TAT).[25,46,47] The human *MCT10* gene is located on chromosome 6q21-q22 and is highly homologous to the *MCT8* gene (49% amino acid identity).[48] In humans, MCT10 is expressed in liver, kidney, intestine, muscle and placenta.[46,47,49] MCT10 is an effective iodothyronine transporter, and also facilitates the cellular uptake and efflux of aromatic amino acids.[25,47,49,50] So far, no patients with mutations in MCT10 have been identified and the polymorphism in the 3'-UTR region of *MCT10* was not associated with TH levels.[51] Although transport of TH across the plasma membrane has been clearly demonstrated, MCT8 and MCT10 also facilitate efflux of TH out of the cell. Therefore it remains unclear if MCT8/MCT10 transported T3 is preferentially directed towards the nucleus thereby increasing the availability of TH for the nuclear receptors, or that a rapid equilibrium is reached near the plasma membrane.

OATPs

OATPs belong to the superfamily of solute carrier organic anion transporters (SLCO). The OATPs identified in humans are classified into 6 families based on their amino acid identity. The proteins are named OATP (Oatp in rodents) followed by the family number, the subfamily letter and a number indicating the individual member within the family. The corresponding genes are named in a similar way: *SLCO* followed by the same number-letter-number combination.[52] OATPs are proteins with 12 transmembrane domains that mediate sodium-independent transport of amphipathic organic compounds,

including TH, steroids, bile salts, drugs and anionic oligopeptides.[48,53–56] Most OATPs are expressed at the basolateral membrane of polarized cells in multiple tissues, including liver, kidney, lung, heart, small intestine, placenta, testis, the blood-brain barrier (BBB) and choroid plexus.[54,57–59] Certain transporters show a more restricted tissue expression pattern, as will be discussed below.

Liver: Oatp/OATPs

In humans, OATP1B1 (*SLCO1B1)* and OATP1B3 (*SLCO1B3)* are considered to be liver-specific and are localized at the sinusoidal membrane of hepatocytes. OATP1B1 is expressed throughout the lobule, while OATP1B3 is mainly expressed around the central vein.[52,57,60–62] The selective expression in human liver suggests that OATP1B1 and OATP1B3 play an important role in the hepatic clearance of albumin-bound amphipathic organic compounds. When expressed in *Xenopus laevis* oocytes, OATP1B1 and OATP1B3 show transport of bile salts (e.g. taurocholate (TCA)), conjugated and unconjugated bilirubin, bromosulfophthalein (BSP), steroid conjugates, several drugs and the thyroid hormones T3 and T4.[55,61,62] OATP1B1 and OATP1B3 have a single rodent orthologue, Oatp1b2 (*Slco1b2*). In *X. laevis* oocytes, the transport properties of Oatp1b2 are very similar to the transport characteristics of OATP1B1 and OATP1B3.[57,62,63] In humans, the tissue distribution of OATP1A2 (*SLCO1A2*) includes the BBB, distal nephron, ciliary body and liver. In human liver, OATP1A2 is expressed in cholangiocytes and in *X. laevis* oocytes it transports T4, T3 and reverse T3 (rT3) beside bile salts, organic anions and cations.[55,64–66] OATP1A2 has four mouse homologs, including Oatp1a1, Oatp1a4, Oatp1a5 and Oatp1a6. In mouse liver, Oatp1a4 is higher expressed in females than in males, the opposite of Oatp1a1.[55,67–70] Hyperthyroidism downregulates mRNA expression of Oatp1a1, Oatp1a4 and Oatp1b2.[16,71,72] Several Oatp-knockout mice have been generated to define the function of the transporter. Oatp1b2 null mice show a normal development. However, in adult mice serum bilirubin levels are increased.[71,73,74] In addition, it was observed that in transporter-deficient mouse models compensatory changes in the expression of other transporters occur, that contribute to the phenotype of the model. To minimize the likelihood of compensatory function by another Oatp superfamily member, an Oatp1a/1b knockout mouse was generated. In these mice, serum levels of conjugated bilirubin and unconjugated bile acids are markedly increased indicating that Oatp1a/1b transporters are essential for the hepatic uptake of these substrates in mice.[75]

SLC10

In humans, the solute carrier transporter family 10 (*SLC10*) represents 7 genes that encode proteins of 248–477 amino acids. The SLC10 family includes the sodium taurocholate cotransporting polypeptide (NTCP, *SLC10A1*), the apical bile salt transporter (ASBT, *SLC10A2*), *SLC10A3–5*, the Na^+-dependent organic anion transporter (SOAT, *SLC10A6*) and *SLC10A7*. Only 3 members (*SLC10A1*, *SLC10A2* and *SLC10A6*) show Na^+-dependent transport of substrates, like bile salts. NTCP is expressed at the basolateral membrane of hepatocytes, while ASBT is expressed at the apical membrane of cholangiocytes, ileum and renal proximal tubules. SOAT, expressed in adrenal gland, testis and placenta, can transport sulfated steroid metabolites and sulfated taurolithocholate. *SLC10A3*–5 and *SLC10A7* are considered orphan transporters, since they show no transport activity.[22,76,77]

Liver: Ntcp/NTCPs

Human NTCP and rodent Ntcp (*SLC10A1/Slc10a1*) show a 77% amino acid identity and code for proteins of 349 and 362 amino acids with 7 putative transmembrane domains. NTCP/Ntcp plays an important role in the enterohepatic circulation of bile salts. Bile salts are the major constituents of bile and are required for systemic energy homeostasis. In addition, bile salts are important for the intestinal digestion of fat and absorption of fat-soluble vitamins. NTCP mediates the uptake of bile salts into hepatocytes and the export of bile salts across the canalicular membrane occurs by the ATP-dependent canalicular bile salt export pump BSEP.[22,78–80] Hepatocellular bile salt uptake by NTCP is driven by the Na^+-gradient maintained by the basolateral Na^+, K^+-ATPase together with the negative intracellular potential.[81,82]

NTCP is capable of transporting both conjugated as unconjugated bile acids in a Na^+-dependent manner, while OATPs have a preference for (Na^+-independent) transport of unconjugated bile acids. In mice, inactivation of the *Slco1a/1b* subfamily resulted in normal serum levels of conjugated bile salts and highly elevated levels of unconjugated bile acids. However, *Slco1b2* KO mice have slightly elevated levels of unconjugated bile acids with normal bile salt levels in serum. These observations suggest that Ntcp is a poor transporter for unconjugated bile acids in mice.[75,83] In conditions of hepatocellular bile acids accumulation, such as cholestasis, the expression of Ntcp is strongly down-regulated. This is considered a hepatic defense mechanism to the overload of potentially toxic bile acids.[79,84,85] Besides its role in bile acids homeostasis, Ntcp is also able to transport sulfated steroids, thyroid hormones, and xenobiotics.[21,86]

Beside their role in the enterohepatic circulation of bile salts, liver-specific transporters such as OATPs and NTCP, are important in the maintenance of lipid and energy

homeostasis by TH. It is necessary to gain insight in the characteristics of these different transporters since liver-specific thyromimetics, of which Eprotirome and Sobetirome (GC-1) are the best studied in humans, are interesting compounds that can have great benefits on for example dyslipidemia, metabolic syndrome and hepatic steatosis without systemic side effects.

PDZ domains

Many members of the OATP/Oatp family have a highly conserved PDZ consensus-binding motif in their C-terminus. PDZ is an acronym combining the first letters of three proteins (**P**ostsynaptic density protein PSD95, Drosophila septate junction protein **d**iscs-large Dlg1, and the epithelial **z**onula occludens-1 protein ZO-1), which were first described to share the domain. The PDZ domain is a structural domain of 80–90 amino acids found in signaling proteins. PDZ domains mediate specific protein-protein interactions and proteins containing PDZ domains play a role in protein complex assembly, in particular at cellular membranes. Based on crystal structures of known PDZ domains, three classes of PDZ consensus binding sites have been defined. PDZ consensus sites that are present in the hepatic OATP/Oatps are all of Class 1, defined by the sequence X-S/T-X-φ, where X represents any amino acid and φ is a hydrophobic amino acid. Despite the large number of described PDZ proteins, it is hard to predict which if any will bind a particular protein with a PDZ consensus-binding site.[87–89] It has been shown that expression of rat Oatp1a1 at the plasma membrane requires interaction with PDZK1, a 70-kDa protein with 4 independent PDZ domains.[89]

Thyroid hormone metabolism: deiodination

Intracellular TH concentrations are accurately controlled by 3 deiodinating selenoenzymes, each with a distinct substrate preference, tissue expression and mode of action. Table 1 illustrates the specific characteristics of the deiodinases type 1 (D1), type 2 (D) and type 3 (D3).

D1 and D2 convert the prohormone T4 to bioactive T3 by outer ring deiodination, while D3 inactivates T4 to rT3 and T3 to 3,3'-T2 by inner ring deiodination.[90,91]

D1 is expressed in liver, kidney, thyroid and pituitary. In particular hepatic D1 activity is considered to contribute to serum T3 levels by converting T4 to T3. Evidence for this includes the observation that both hepatic D1 activity and plasma T3 levels decrease in conditions such as fasting and critical illness.[92] On the other hand, D1 seems to be positively regulated by T3 and D1KO mice have normal serum T3 concentrations.[93] In

Table 1. Characteristics of human iodothyronine selenodeiodinases. (Adapted from Bianco and Kim, J Clin Invest. 2006)

	D1	D2	D3
Biochemical properties			
Molecular weight (kDa)	29	30.5	31.5
Preferred substrate	rT3	T4, rT3	T3, T4
K_m	10^{-7}	10^{-9}	10^{-9}
Half-life	Hours	± 20 min	Hours
Subcellular localization	Plasma membrane	Endoplasmic reticulum	Plasma membrane
Susceptibility to PTU	High	Low	Low
Tissues with high activity	Liver, kidney	CNS, pituitary, brown adipose tissue, placenta	CNS, placenta
Response to elevated T3 & T4			
Transcriptional	↑ ↑	↓	↑ ↑
Translational	?	↓ ↓ ↓ (ubiquitination)	?
Physiological role	rT3 clearance	Thermogenesis, development, to provide intracellular and plasma T3	T3 and T4 clearance
Physiological regulation			
Induction	T3	Cold exposure, catecholamines	Tissue injury, T3
Repression	Fasting, illness	T3	Glucocorticoids

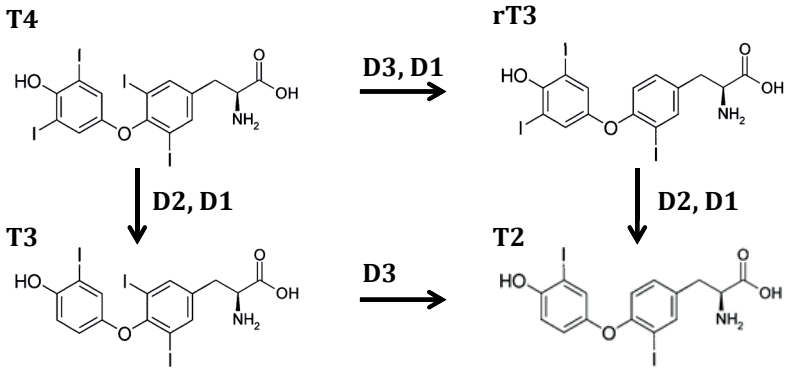

Figure 3. Deiodinase reactions. Adapted from Bianco and Kim, J Clin Invest. 2006

addition, D1 shows a higher catalytic efficiency for rT3 compared to T4. These observations suggest that D1 could play a more important role in recycling iodide from inactive iodothyronines for reuse in the thyroid gland. No human mutations in *DIO1* have been reported yet.

D2 is expressed in the central nervous system, pituitary, skeletal muscle, brown adipose tissue and the reproductive tract, where it regulates local TH homeostasis.[94–96] D2 func-

tions as a TH activating enzyme, however it is unclear to what extent D2 contributes to serum T3 levels. The observation that D2 is upregulated by hypothyroidism and downregulated in hyperthyroidism, illustrates that thyroid status controls D2 activity and vice versa, changes in D2 activity contribute to the plasma and cellular levels of T3. The expression of D2 is inversely regulated by T4 and rT3, by induction of ubiquitination at the posttranslational level.[97] However, D2KO mice show normal serum T3 concentrations. Serum T4 and TSH levels in these mice are increased, suggesting that the pituitary of D2KO mice is resistant to the feedback effect of plasma T4.[90,91,98] In addition, D2KO mice display only a mild neurological phenotype despite strongly decreased brain T3 levels. This favors the suggestion that important compensatory mechanisms in brain must be involved in the absence of D2.[99] To date, no mutations in the human *DIO2* gene have been described. However, patients with mutations in the SECIS-binding protein SBP2, showed decreased *DIO2* enzymatic activity in combination with decreased serum T3 concentrations and increased T4, rT3 and TSH levels. SBP2 is important for the synthesis of selenoproteins, such as the deiodinases.[100,101]

D3 is an inactivator of TH and serves as a modulator of intracellular TH levels by catalyzing the conversion of the bioactive T3 and T4 into the inactive T2 and rT3 metabolites. *DIO3* is subject to genomic imprinting and is preferentially expressed from the paternal allele. D3 is highly expressed in fetal tissues, where it is responsible for low TH levels during early development. D3KO mice show neonatal mortality, growth retardation and abnormal serum TH concentrations. In adult life, D3 activity is only detectable in central nervous system, skin and placenta.[102] In other tissues, e.g. liver, skeletal muscle, D3 can be re-activated in conditions of critical illness and injury.[103,104] As for the other deiodinases, no mutations in *DIO3* have been identified so far.

TH action

An optimal intracellular TH concentration is required for TH action, which is mediated by binding of the bioactive T3 to nuclear TH receptors (TRs). TRs belong to the nuclear receptor superfamily of ligand-inducible transcription factors. Preferentially, TRs bind to TH response elements (TREs) in target gene promoters as heterodimers with retinoid X receptors (RXRs), thereby regulating target gene expression. In the absence of TH, unliganded TRs recruit corepressors (e.g. nuclear receptor corepressor [NCoR], silencing mediator for retinoic acid and thyroid receptors [SMRT]) and histone deacetylase (HDAC), thereby repressing basal transcription of genes that are positively regulated by TH. Binding of T3 to TRs results in dissociation of the corepressor complex and recruitment of coactivator proteins (e.g. steroid receptor coactivator 1 [SRC-1], CREB-binding protein [CBP], and CBP-associated factor [pCAF]), which induce transcriptional activation.[7,105–107]

In humans, TRs are encoded by two genes (*THRA* and *THRB*) on chromosomes 17 and 3, respectively. By alternative splicing two different isoforms, which differ in their carboxy-terminal regions, are generated from the *THRA* locus: TRα1 is ubiquitously expressed in the central nervous system (CNS), bone, heart, skeletal muscle and gastrointestinal tract while the non-T3 binding isoform TRα2 is more widely expressed (e.g. brain and testis). From the *THRB* locus two receptor proteins are generated: TRβ1 and TRβ2, which differ in their amino-terminal regions. TRβ1 is considered the major isoform in liver, kidney and thyroid. TRβ2 has a more restricted expression pattern regulating neurosensory development as well as the HPT-axis.[67,108,109] Since 1986 it has been known that heterozygous mutations within three hotspots of the ligand-binding domain (LBD) of TRβ result in resistance to thyroid hormone β (RTHβ), a clinical syndrome that was recognized two decades earlier as refractoriness to TH. The incidence of RTHβ is estimated 1 in 40,000 and numerous mutations have been identified so far. Patients with RTHβ are characterized by elevated serum TH levels, a non-suppressed TSH, and a variable phenotype comprising goiter, tachycardia and increased energy expenditure.[7,110] Human TRβ and TRα are highly homologous, in particular in their hormone binding domains, showing an overall 80% amino acid identity. Taking into account the large number of different TRβ mutations associated with RTHβ, the identification of mutations in TRα had been anticipated. In an attempt to predict the clinical consequences of a defective TRα, several knockin and knockout mouse models have been generated. Interestingly, mice harboring different, heterozygous TRα mutations are viable and exhibit recognizable abnormalities. However these mice have a near-normal thyroid function.[111–114] Due to the absence of a clear biochemical thyroid phenotype, no human mutation in *THRA* was reported until 2012.

Outline of the thesis

The work in this thesis focuses on the molecular mechanisms of TH action at the level of transporters and receptors. Hereby, we report on various aspects of thyroid hormone and thyroid hormone analogs transport in liver. In addition, we describe a wide spectrum of RTHα phenotypes and elaborate on the role of TRα in human erythropoiesis.

In **chapter 2** we investigated to what extent the thyroid hormone transporters MCT8 and MCT10 modulate T3 availability for binding to its thyroid hormone receptor in the cell nucleus and its metabolism by type 3 deiodinase at the cell periphery.

In **chapter 3** and **chapter 4** we explored the characteristics of different human and mouse liver-specific thyroid hormone transporters (OATPs and NTCPs) and compared their role in the transport of thyroid hormone and thyroid hormone analogs such as Eprotirome and GC-1.

In **Chapter 5** we report on the youngest patient identified with RTHα so far, which was treated with levothyroxine from very young age.

In **chapter 6** we describe a total of 10 RTHα patients with three different mutations in TRα. This large case series enables us to report on the diversity in the RTHα phenotype and compare the phenotypes of both affected and non-affected individuals from a family with the same mutation.

In **chapter 7** we report on the role of TRα in erythropoiesis and the pathogenesis of anemia, which seems to be a frequently appearing characteristic in RTHα.

In **chapter 8** we present an overview of the clinical and biochemical characteristics of all patients identified with RTHα to date. Here we discuss the genetic basis and molecular pathogenesis of the disorder together with the effects of levothyroxine treatment.

In **chapter 9** we discuss the findings presented in this thesis in view of the current literature and we explore possible implications of these studies.

EFFECTS OF MCT8 AND MCT10 ON NUCLEAR ACTIVITY OF T3

2

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Journal of Molecular and Cellular Endocrinology 2016

Abstract

Transport of thyroid hormone (TH) across the plasma membrane is necessary for the genomic action of T3 mediated by its nuclear T3 receptor. MCT8 and MCT10 have been identified as important TH transporters. Mutations in MCT8 result in severe psychomotor retardation. In addition to TH transport into the cell, MCT8 and MCT10 also facilitate TH efflux from cells. Therefore, the aim of this study was to examine if MCT8 and MCT10 increase the availability of T3 for its nuclear receptor rather than generate a rapid equilibrium between cellular and serum T3.

T3 action was investigated in JEG3 cells co-transfected with TRβ1 and a T3 response element-driven luciferase construct, and T3 metabolism was analyzed in cells transfected with type 3 deiodinase (D3). In addition, cells were transfected with MCT8 or MCT10 and/or the cytoplasmic T3-binding protein mu-crystallin (CRYM). Luciferase signal was markedly stimulated by incubating cells for 24 h with 1 nM T3, but this response was not augmented by MCT8 or MCT10 expression. Limiting the time of T3 exposure to 1–6 hours and co-transfection with CRYM allowed for a modest increase in luciferase response to T3. In contrast, T3 metabolism by D3 was potently stimulated by MCT8 or MCT10 expression, but it was not affected by expression of CRYM.

These results suggest that MCT8 and MCT10 by virtue of their bidirectional T3 transport have less effect on steady-state nuclear T3 levels than on T3 levels at the cell periphery where D3 is located. CRYM alters the dynamics of cellular TH transport but its exact function in the cellular distribution of TH remains to be determined.

Introduction

Lack of thyroid hormone (TH) in early life leads to severe neurological dysfunction.[1,115,116] TH regulates gene expression via binding of the biological active hormone T3 to its nuclear receptor (TR), which is bound to a T3 response element (TRE) in the promotor region of target genes. To mediate its effects, TH must first be transported across the plasma membrane by specific transporters. Several TH transporters from different families have been identified, such as organic anion transporting polypeptides (OATP), bile acid transporters, L-type amino acid transporters (LAT) and monocarboxylate transporters (MCT).[117] Two highly homologous monocarboxylate transporters, MCT8 and MCT10, are so far the only identified transporters with a high activity and specificity for TH.[14,24,25,118]

MCT8 is expressed in neurons and at the blood-brain barrier.[35,36,42,44,119–121] Other expression sites are the heart, placenta, kidney, liver and the adrenal glands.[29,42] Inactivating mutations in MCT8 result in the X-linked Allan-Herndon-Dudley syndrome (AHDS) characterized by severe psychomotor retardation and abnormal serum TH levels (high serum T3, low T4 and normal TSH levels).[30,31,122,123] Patients with AHDS also suffer from hypotonia, muscular hypoplasia, and a delayed development and myelination.[122,124] The complete pathogenesis of AHDS is not yet completely understood, but it is hypothesized that AHDS is due to a hypothyroid state of the MCT8-expressing neurons as a result of the inactivating mutations in MCT8.[35–38,124] In contrast to humans, MCT8 KO mice do not show a neurological phenotype, although they have very similar abnormal serum TH levels as AHDS patients and low T3 levels in the brain.[26,39,42] The absence of brain abnormalities in MCT8 KO mice may be explained by the more abundant expression of OATP1C1 at the blood-brain barrier in mice than in humans.[44,125]

MCT10, the highly homologous family member of MCT8, is expressed in multiple tissues, including intestine, kidney, liver, muscle, and placenta[37,47] (Nishimura and Naito, 2008). There is little expression of MCT10 in the brain.[126] In contrast to MCT8, no patients with mutations in MCT10 have been identified so far. Apart from TH, MCT10 is also capable of transporting amino acids.[25]

MCT8 and MCT10 facilitate not only the cellular uptake but also the efflux of TH from cells. It is therefore unclear if MCT8/MCT10 transported T3 is preferentially directed towards the nucleus, thereby increasing the availability of TH for its nuclear receptors, or that it results in a rapid equilibrium near the plasma membrane. We therefore investigated if the presence of MCT8 and MCT10 has an effect on directing T3 to the nucleus, thereby increasing the availability of T3 for its nuclear receptors. This was done by analyzing the effects of the overexpression of MCT8 or MCT10 on the TRβ1-mediated stimulation of a TRE-driven luciferase reporter by T3 in JEG3 human choriocarcinoma cells, which show low endogenous expression of TH transporters (Capri, Friesema, Kersseboom et

al., 2013). The findings were compared with the effects of MCT8 and MCT10 on the metabolism of T3 by type 3 deiodinase (D3) located in the plasma membrane.[127] In addition, we studied the effects of the cytoplasmic TH-binding protein μ-crystallin (CRYM) on the availability of T3 for binding to its receptor in the cell nucleus and its metabolism by D3 at the cell periphery.

Material and methods

Materials

Unlabeled T3 was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands [NL]). $Na^{125}I$ was obtained from Perkin Elmer (Groningen, NL). [3'-^{125}I]T3 was prepared as previously described.[128]

Plasmids

Human MCT8,[24] MCT10,[25] CRYM,[25] and D3[24] plasmids were obtained as previously described. hTRβ1 was cloned into the pcDNA3.1 expression vector by Dr. W.M. van der Deure (Erasmus MC, Rotterdam, NL). N-terminal Flag-tagged hTRβ1 with an optimized Kozak translation start site was also constructed in pcDNA3. The pdV-L1 construct containing a T3 responsive firefly luciferase (LUC) reporter and a control renilla luciferase (REN) reporter was kindly provided by Dr. W.S. Simonides (VU Medical Center, Amsterdam, NL).[129] Both LUC and REN genes are driven by the SERCA1 minimal promoter, and two T3 response elements (TREs) are inserted in front of the minimal promoter of the LUC gene.

Cell culture

JEG3 cells were cultured at 37 C and 5% CO2 in DMEM/F12 medium (Invitrogen, Bleiswijk, NL) supplemented with 9% heat-inactivated fetal bovine serum (FBS) and 100 nM Na_2SeO_3 (Sigma-Aldrich).

[^{125}I]T3 transport

JEG3 cells were cultured in 24 or 96-well plates and transiently transfected with various combinations of pdV-L1, hTRβ1 or Flag-hTRβ1, hMCT8 or hMCT10, hCRYM, and/or hD3, and the total amount of plasmid was brought to 60–75 ng (96 wells) or 200 ng (24 wells) with empty pcDNA3. Transfection was performed according to the manufacturer's protocol using FuGene6 or X-tremeGENE9 (Roche, Almere, NL). In some experiments, cells were washed and incubated for 24 hours with 500 μl DMEM/F12 containing charcoal-stripped FBS prior to transfection. Two days after transfection, cells were washed twice with 200 μl (96 wells) or 500 μl (24 wells) DMEM/F12 + 0.1%BSA and

incubated for different time periods at 37 C and 5% CO2 with 100 µl (96 wells) or 500 µl (24 wells) DMEM/F12 + 0.1%BSA with 1 nM T3 and 10,000 cpm (96 wells) or 50,000 cpm (24 wells) [^{125}I]T3. After incubation, the cells were again washed, lysed with 0.1 M NaOH, and counted for radioactivity.

Intact cell T3 metabolism

JEG3 cells were transfected as described above for the uptake assay in 96 well plates. Incubations were performed for different time periods at 37 C and 5% CO2 with 1 nM T3 and 100,000 cpm [^{125}I]T3 in 100 µl DMEM/F12 + 0.1%BSA. Next, 100 µl medium was added to 100 µl ice-cold ethanol. The mixtures were incubated for 30 minutes on ice, and then centrifuged for 15 minutes at 3500 rpm and 4 C. Finally, 100 µl supernatant was mixed with 100 µl 0.02M ammonium acetate (pH 4.0), and the samples were analyzed by HPLC as previously described.[24]

Luciferase activity

Two days after transfection, cells were incubated for the different time periods with 1 nM T3. After washing and replacement with medium without T3, incubations were continued for a total time of 24 hours. LUC and REN activities were determined as previously described.[130] T3 has only a small effect on REN activity compared with its effect on LUC expression. Results are presented as LUC/REN ratio but are not essentially different from the absolute LUC activity.

Statistical tests

All experiments were performed in triplicate and presented as mean ± SEM.of n experiments. Statistical differences were calculated using Student's t-test or a 2-way ANOVA followed by Bonferroni post-hoc test, as indicated in the figure legends. $P < 0.05$ was considered significant.

Results

The transcriptional activity of T3 was studied using JEG3 cells transfected with human TRβ1 and a construct expressing LUC under control of a TRE-dependent promoter and REN under control of a constitutive promoter. Initial experiments were conducted under steady-state conditions, where cells were incubated for 24 hours without or with 1 nM T3. As demonstrated before using TRα1,[130] this resulted in a marked stimulation of the LUC/REN ratio. To investigate if MCT8 or MCT10 increase the nuclear availability of T3, the effect of 24-hour incubation with 1 nM T3 was also studied in cells co-transfected with plasmids coding for MCT8 or MCT10. Remarkably, this did not result in any further

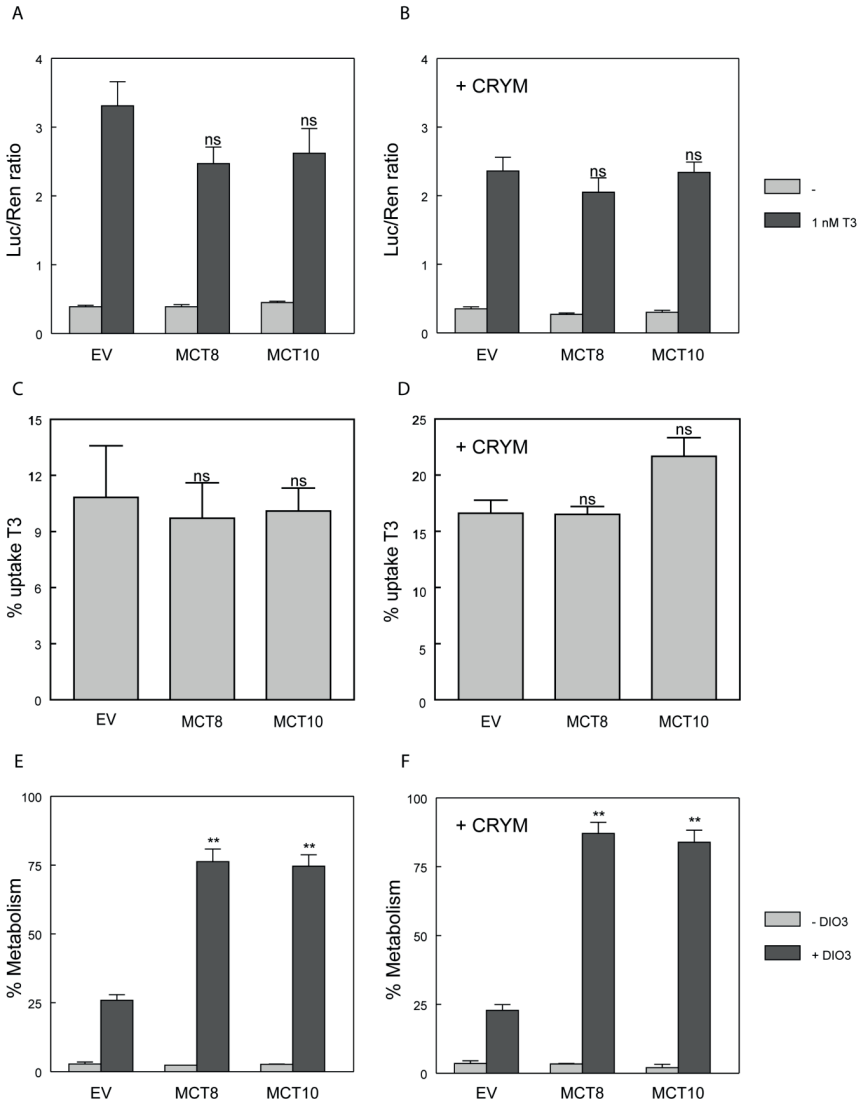

Figure 1.

(A, B) Functional analysis of JEG3 cells co-transfected with a TRE-regulated LUC and control REN construct and TRβ1 in combination with either empty vector, MCT8 or MCT10, without **(A)** or with **(B)** CRYM. Cells were incubated for 24 hours without or with 1 nM T3. Values represent means ± SEM of 5 experiments performed in triplicate. ns, not significant vs. cells without transporter.

(C, D) 24-hour uptake of 1 nM [125I]T3 in JEG3 cells in the absence **(C)** or presence **(D)** of CRYM. Cells were transiently transfected with empty vector, MCT8 or MCT10. Values represent means ± SEM of 3 experiments performed in triplicate. ns, not significant vs. cells without transporter.

(E, F) 24-hour metabolism in JEG3 cells of [125I]T3 in the absence **(E)** or presence **(F)** of CRYM. Cells were transfected with empty vector, MCT8 or MCT10, with or without D3. Values represent means ± SEM of 2 experiments performed in triplicate; ** $P < 0.01$ vs. cells without transporter.

increase in the T3-stimulated LUC/REN ratio compared with control cells transfected with empty plasmid (Figure 1A).

Cellular uptake of 1 nM [^{125}I]T3 was determined in parallel 24-hour incubations, showing that transfection with MCT8 or MCT10 did not stimulate cellular T3 accumulation under these steady-state conditions (Figure 1C). This is in keeping with previous observations that MCT8 and MCT10 facilitate both cellular T3 influx and efflux. Therefore, expression of MCT8 or MCT10 has little effect on the steady-state cellular T3 concentration in the presence of other, endogenous transporters. It has also been shown previously that expression of the cytoplasmic T3 binding protein μ-crystallin (CRYM) markedly augments the cellular accumulation of T3 induced by MCT8 or MCT10 during short incubations. However, co-transfection with CRYM did not enable MCT8 or MCT10 to stimulate the transcriptional activity (Figure 1B) or cellular uptake (Figure 1D) of T3 in these prolonged incubations.

Little metabolism of T3 was observed during 24-hour incubation with JEG3 cells transfected with empty vector, MCT8 or MCT10 alone or in combination with CRYM (Figure 1E,F). About 25% of added T3 was metabolized in parallel incubations with cells transfected with D3 alone or in combination with CRYM. Co-transfection of MCT8 or

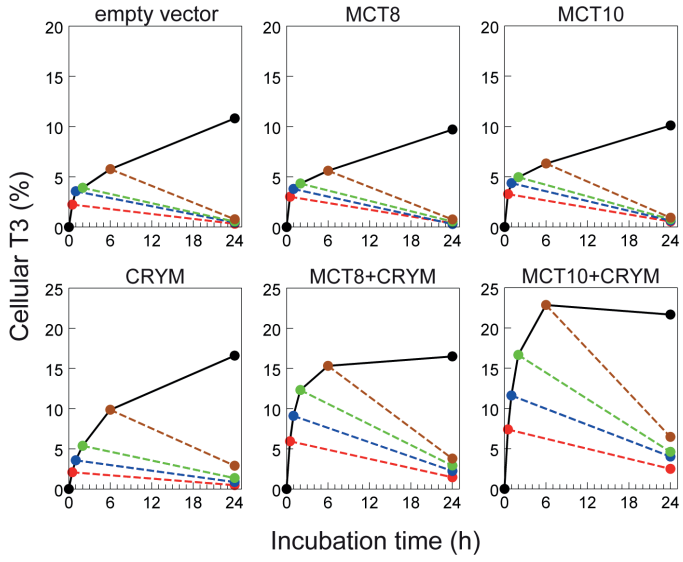

Figure 2.

Cellular accumulation of [125I]T3 in cells transfected with empty vector, MCT8 or MCT10 without or with CRYM as function of incubation time (0.5–24 hours). Interrupted lines indicate the loss of cellular T3 when [125I]T3-containing medium is replaced at the indicated time points with T3 deplete medium and the incubation continued for a total period of 24 hours. Results are the means of 2 experiments performed in triplicate.

MCT10 resulted in a similar, marked stimulation of T3 metabolism, amounting to ~75% and ~90% of added T3 in the absence or presence of CRYM, respectively (Figure 1E,F).
Since expression of MCT8 or MCT10 in the absence or presence of CRYM did not have a significant effect on the steady-state cellular and nuclear accumulation of T3 during prolonged incubations, we decided to test shorter incubation times. Therefore, JEG3 cells transfected with the various constructs were incubated for 0.5–24 hours with 1 nM T3; after replacing the medium with T3 deplete medium the incubation was continued until a total period of 24 hours to allow sufficient synthesis of reporter activity. Figure 2 shows that in the absence of CRYM, expression of MCT8 or MCT10 stimulated the cellular accumulation of T3 only during the first 30 minutes of incubation.
However, in the presence of CRYM, expression of MCT8 or MCT10 resulted in the marked stimulation of T3 uptake during at least 6 hours of incubation. Therefore, we decided to test the effects of MCT8 and MCT10 in the absence or presence of CRYM on the uptake, nuclear activity and D3-mediated metabolism during a 4-hour incubation with T3. In addition, the effects of co-transfection with D3 were studied on the cellular accumulation and transcriptional activity of T3. Figure 3A shows that expression of MCT8 or MCT10 in the absence of CRYM had no significant effect on the cellular accumulation of T3 during 4 hours of incubation.
Transfection of cells with CRYM alone did not markedly stimulate T3 uptake over the 4-hour period (Figure 3B). However, transfection of CRYM-expressing cells with MCT8 or MCT10 stimulated cellular T3 accumulation 2 and 3-fold, respectively. Expression of MCT8 or MCT10 in the absence of CRYM did not have a significant effect on the transcriptional activity induced by exposure of the cells for 4 hours to T3 (Figure 3C). Transfection of the cells with CRYM alone resulted in a significant decrease in nuclear T3 activity (Figure 3D). However, transfection of CRYM-expressing cells with MCT8 or MCT10 resulted in the stimulation of the transcriptional activity of T3 to levels at or above those observed in cells expressing MCT8 or MCT10 in the absence of CRYM.
In these short incubations, T3 metabolism was again negligible in cells transfected with empty vector, MCT8, MCT10 or CRYM alone (Figure 3E,F). About 5% of added T3 was metabolized during 4-hour incubation with cells expressing D3 in the absence or MCT8 or MCT10, and this was increased to about 30% after co-transfection with MCT8 or MCT10. The metabolism of T3 by D3 in MCT8 or MCT10-expressing cells was not affected by co-transfection of CRYM. Expression of D3 reduced the cellular uptake and transcriptional activity of T3 independent of the expression of CRYM and completely prevented the stimulation of T3 uptake and nuclear activity induced by MCT8 or MCT10 observed in the presence of CRYM (Figure 3E,F).
Finally, the transcriptional activity induced by incubation of the variously transfected cells for different time periods with 1 nM T3 was studied as a function of time-integrated cellular T3 concentration. By plotting the percentage of T3 uptake against time, the

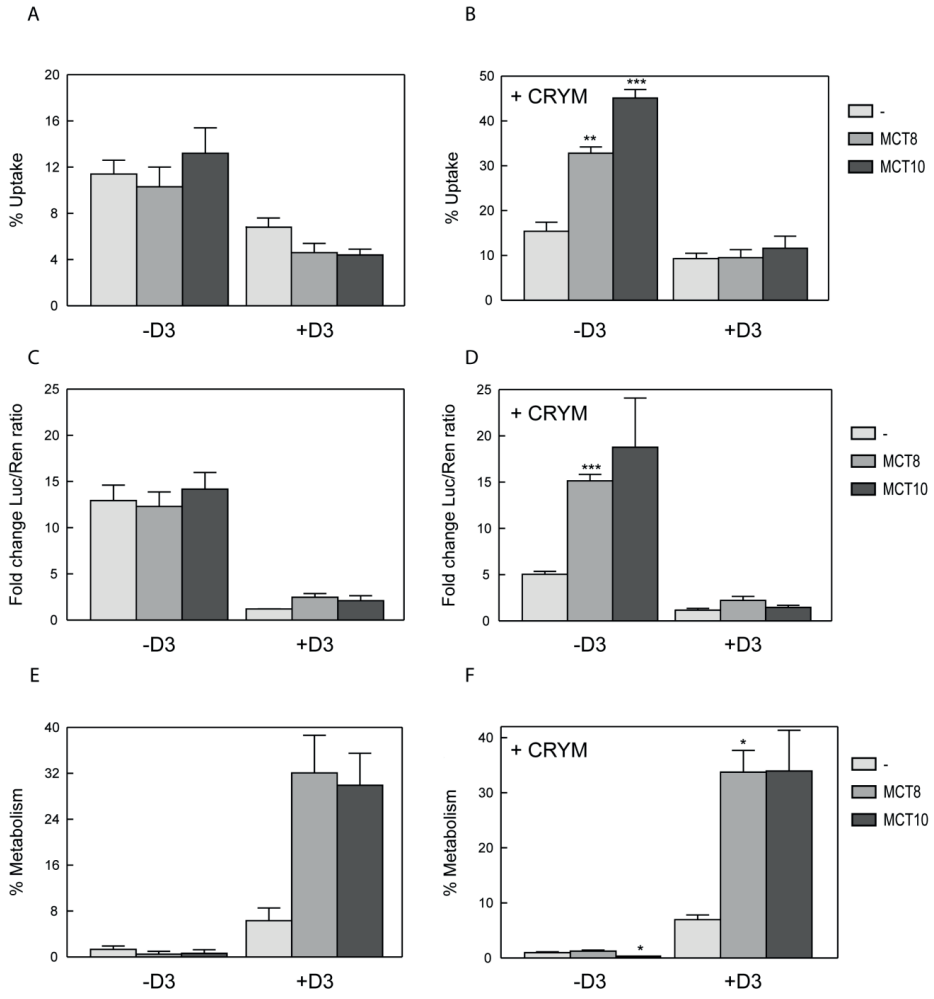

Figure 3.
Cellular uptake **(A,B)**, nuclear activity **(C,D)** and metabolism **(E,F)** of 1 nM T3 incubated for 4 hours with JEG3 cells co-transfected with reporter construct and Flag-TRβ1 plus MCT8, MCT10, CRYM and/or D3. In **(C)** and **(D)**, incubations were continued with T3 deplete medium until 24 hours to allow sufficient time for luciferase synthesis. Results are the means ± SEM of 2–4 experiments performed in triplicate; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. cells without transporter.

area under the curve represents the intracellular T3 concentration during the whole incubation period (Figure 2A). There was a good correlation between the integrated cellular T3 concentration and the luciferase response to T3, both in the absence and presence of CRYM. However, the intracellular T3 concentrations associated with similar T3 responses were higher in the presence than in the absence of CRYM (Figure 4).

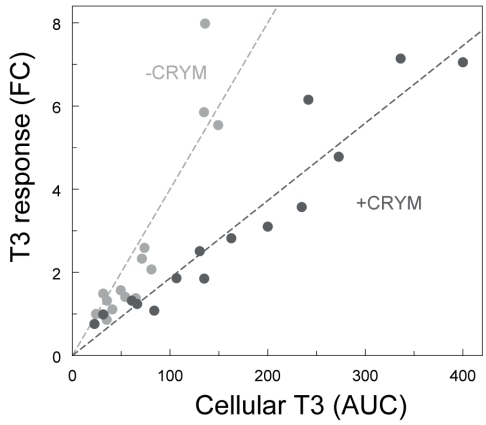

Figure 4.
T3-stimulated reporter activity as a function of T3 accumulation in JEG3 cells transfected with reporter construct and TRβ1 plus empty vector, MCT8, MCT10, and/or CRYM. Cells were incubated for 0.5–24 hours with 1 nM T3 and subsequently until a total period of 24 hours in the absence of T3 to allow sufficient luciferase synthesis. The experiment was performed in triplicate. FC: fold change, AUC: area under the curve.

Discussion

In the current study we demonstrate that transfection of JEG3 cells with MCT8 or MCT10 has widely different effects on the binding of T3 to the nuclear receptor compared with its deiodination by D3 located in the cell membrane.

A schematic overview of the data is presented in Figure 5. Figure 5A represents a non-transfected JEG3 cell where cellular T3 uptake and efflux is mediated by endogenous transporters. In MCT8 or MCT10 overexpressing cells, both T3 influx and efflux are increased. Therefore, steady-state intracellular T3 levels and thus nuclear receptor binding are hardly affected (Figure 5B). In D3 expressing cells, T3 is degraded to 3,3-T2, resulting in decreased nuclear T3 receptor activation (Figure 5C). Transfection of D3 expressing cells with MCT8 or MCT10 stimulates T3 metabolism but does not increase nuclear availability of T3 (Figure 5D). The cytoplasmic binding protein CRYM decreases T3 efflux and thus enhances the transient increase in cellular T3 accumulation in MCT8 or MCT10 overexpressing cells. Consequently, nuclear receptor activation is stimulated by expression of MCT8 or MCT10 during short T3 exposure times (Figure 5E). However, co-transfection of D3 completely prevents T3 action even in the presence of CRYM and MCT8 or MCT10, suggesting that cellular T3 is degraded before it can reach the nuclear receptor (Figure 5F).

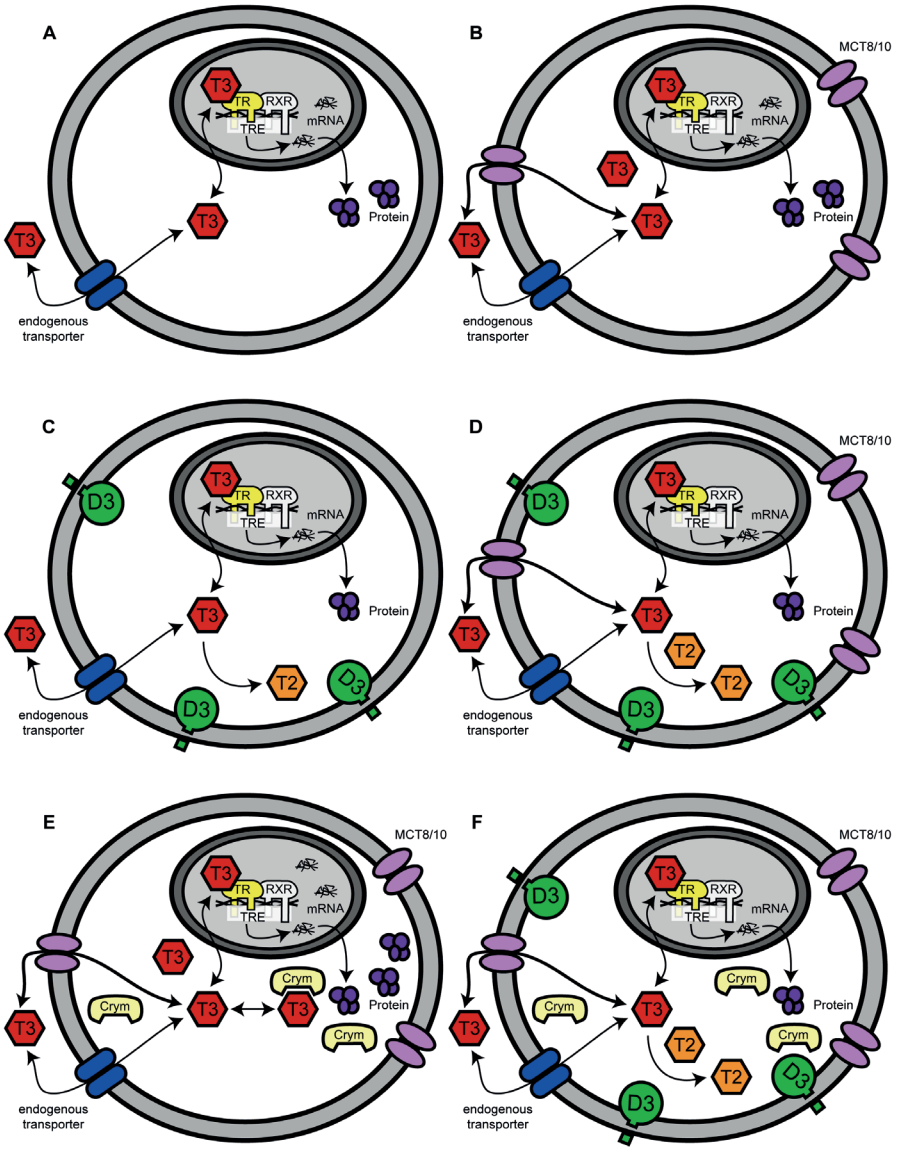

Figure 5.
Schematic representation of a TH target cell with overexpression of MCT8/10, CRYM and/or D3. T3 responsive gene expression is controlled by a positively regulated TRE.

Inactivating mutations in MCT8 lead to AHDS.[122,123] MCT10, the highly homologous family member of MCT8, has been studied in less detail and no patients with mutations in MCT10 have yet been identified.

Overexpression of MCT8 or MCT10 stimulates T3 uptake, resulting in a rapid rise in intracellular T3 concentration, as we have shown previously.[24,25] Nevertheless, the increased uptake results in only a modest increase in biological activity of T3. This is explained by the fact that both MCT8 and MCT10 are also capable of T3 efflux, leading to a rapid equilibrium between influx and efflux.[24,25] Interestingly, some patients with AHDS have a milder phenotype, which has been ascribed to a greater impact of their mutations on the efflux than on the uptake of TH by MCT8.[131,132]

Co-transfection of either MCT8 or MCT10 greatly stimulates T3 metabolism by D3, confirming our earlier findings.[24] Rapid degradation by D3 reduces the nuclear availability of T3, irrespective of the transfection with MCT8 or MCT10. D3 is mainly present in fetal tissues and its expression level declines after birth.[133,134] A major site of action of D3 is the central nervous system, where it acts primarily in neurons.[102,135] It was recently shown in mice that D3 protects the cerebellum from high TH levels during fetal life and in the neonatal period.[136] Our data suggest that TH transporters can only raise intracellular T3 concentrations when D3 activity is low.

Expression of the intracellular binding protein CRYM inhibits the efflux of T3.[137] This extends the period after addition of T3 during which MCT8 or MCT10 increase intracellular T3 before equilibrium is reached between T3 influx and efflux. We utilized this property of CRYM in our *in vitro* testing system, but the possible physiological roles for CRYM are intriguing. It has homology to enzymes involved in glutamate and ornithine metabolism, and T3 binding to CRYM depends on NADPH and thiol cofactors.[137,138] It has been hypothesized that CRYM may play a role in the delivery of TH to mitochondria or to the nucleus, depending on the redox state of the cytoplasm.[138] CRYM has recently been identified as an enzyme with ketimine reductase activity regulated by TH.[139] The apparent Kd value of T3 for CRYM amounts to 0.3 nM.[140]

CRYM is found in the inner ear, and mutations in CRYM have been associated with non-syndromic deafness.[141] Furthermore, CRYM is abundantly expressed in the central nervous system; in the cerebral cortex predominantly in the cytoplasm of neurons.[142] Co-transfection of cells with MCT8, CRYM and D3 may thus mimic to some extent the handling of TH in central neurons. The co-transfection of CRYM in addition to D3 leads to a similar T3 metabolism as D3 alone. This suggests that the rapid degradation of T3 prohibits an increase in cytoplasmic T3 levels, thus decreasing nuclear T3 availability.

The most remarkable finding in our study is the marked stimulation of intracellular metabolism of T3 by D3 if cells are co-transfected with MCT8 or MCT10. This is in contrast to the modest stimulation of T3 action as detected by the promoter-luciferase construct. D3 is located in the plasma membrane with its active center located on the

cytoplasmic surface.[24,127,143] The biological activity of T3 is mediated by its nuclear receptor. Therefore, the action of T3 requires a much greater penetration into the cell than its deiodination (Figure 5). The extent of T3 distribution within the cell depends on a number of factors, including the rate of T3 transport back to the extracellular milieu and its binding to intracellular proteins that may facilitate its further distribution. Our results suggest that in MCT8 over-expressing cells, efflux of T3 is much more rapid than its transport to the nucleus, explaining why accumulation of T3 in the nucleus is modest despite the strong stimulation of cellular T3 uptake.

In contrast, intracellular metabolism by D3 is greatly stimulated by co-transfection with MCT8 or MCT10 irrespective of the expression of CRYM. This suggests that the available T3 concentration at the D3 active center is much less affected by the rapid T3 efflux mediated by MCT8 or MCT10. We speculate that transporters such as MCT8 or MCT10 that mediate both rapid uptake and efflux of T3 have a much greater effect on intracellular T3 availability at the cell periphery than at the cell nucleus even in the presence of the cytoplasmic high-affinity T3-binding protein CRYM.

It should be realized that JEG3 cells express endogenous TH transporters, which is reflected by the steady increase in T3 uptake even during prolonged incubations with non-transfected cells. Of course, the effects of transfected MCT8 or MCT10 in cells already expressing endogenous transporters are quite different from cells devoid of other transporters. MCT8 may be the only significant TH transporter in certain populations of central neurons where it plays a crucial role in TH metabolism and action during critical periods of brain development.[37] In addition to neurons, MCT8 is importantly expressed in endothelial cells of the blood-brain barrier (BBB). Perhaps, both TH uptake and efflux functions of MCT8 are involved in the transcellular transport of T4 and T3 across the BBB. Indeed MCT8 has been localized in the apical (blood-facing) as well as the basolateral (brain-facing) membranes of the BBB endothelial cells.[44]

In conclusion, we demonstrated that over-expression of MCT8 or MCT10 in JEG3 cells induce only a transient increase in cellular T3 accumulation upon addition of T3, resulting in a modest increase in the nuclear activity of T3. In the presence of CRYM, the temporary effects of MCT8 or MCT10 on cellular accumulation and nuclear activity of T3 are enhanced. However, this effect is annulled in the presence of D3 due to rapid degradation of T3. MCT8 and MCT10 appear to have a much greater effect on the cellular availability of T3 for D3 located in the plasma membrane than for the T3 receptor located in the nucleus. We hypothesize that this differential effect of MCT8 and MCT10 on TH availability for processes located at the cell periphery or at the nucleus are inherent to the rapid cellular TH efflux also mediated by these transporters. The implications of our findings for the pathogenic mechanism of AHDS remain to be explored.

Acknowledgements

This work was supported by an Erasmus MC MRACE grant and a ZonMW-TOP grant (921.12.044) to Robin P. Peeters. Alies A. van Mullem was supported by a grant from the SMILE Foundation and the Sherman Family.
We thank Dr. W.S. Simonides for kindly providing the TRE luciferase construct and Dr. W.M. van der Deure for generating the TRβ1 cDNA construct.

ROLE OF THE BILE ACID TRANSPORTER SLC10A1 IN LIVER TARGETING OF THE LIPID-LOWERING THYROID HORMONE ANALOG EPROTIROME*

3

*Dedicated to the memory of Dr. Chester Ridgway

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Endocrinology 2017

Abstract

The thyroid hormone (TH) analog eprotirome (KB2115; 3-[[3,5-dibromo-4-[4-hydroxy-3-(1-methylethyl)-phenoxy]-phenyl]-amino]-3-oxopropanoic acid) was developed to lower cholesterol through selective activation of the T3 receptor isoform TRβ1 in the liver. Interestingly, eprotirome shows low uptake in non-hepatic tissues, explaining its lipid-lowering action without adverse extra-hepatic thyromimetic effects. Clinical trials have shown marked decreases in serum cholesterol levels.

We explored the transport of eprotirome across the plasma membrane by members of three TH transporter families: monocarboxylate transporters MCT8 and 10, Na-independent organic anion transporters OATP1A2, 1B1, 1B3, 1C1, 2A1 and 2B1, and Na-dependent organic anion transporters SLC10A1-7. Cellular transport was studied in transfected COS1 cells using [^{14}C]eprotirome and [^{125}I]TH analogs.

Of the 15 transporters tested initially, the liver-specific bile acid transporter SLC10A1 (also known as Na/taurocholate co-transporting polypeptide, NTCP) showed the highest eprotirome uptake (>7 fold induction after 60 min) as well as TRβ1-mediated transcriptional activity. Uptake of eprotirome by SLC10A1 was Na^+ dependent and saturable with a Km of 8 μM. Eprotirome transport was inhibited by known substrates for SLC10A1 (*e.g.* cholate and taurocholate), and by TH analogs such as triiodothyropropionic acid and triiodothyroacetic acid. However, no significant SLC10A1-mediated transport was observed of these [^{125}I]TH analogs. To further explore the role of SLC10A1 in the liver targeting of eprotirome, we studied the plasma disappearance and biliary excretion of [^{14}C]eprotirome injected in control and Slc10a1 knockout mice. Although eprotirome is also transported by mouse Slc10a1, the pharmacokinetics of eprotirome were not affected by Slc10a1 deficiency.

In conclusion, we have demonstrated that the liver-specific bile acid transporter SLC10A1 effectively transports eprotirome. However, Slc10a1 does not appear to be critical for the liver targeting of this TH analog in mice. Therefore, the importance of SLC10A1 for liver uptake of eprotirome in humans remains to be elucidated.

Introduction

Hypercholesterolemia is associated with atherosclerosis and thus represents a major health risk for ischemic heart disease and stroke, the foremost causes of death in middle and high income countries [144]. Thyroid hormone (TH) plays an important role in the regulation of different steps in lipid metabolism such as lipolysis and hepatic cholesterol clearance [145]. These effects are importantly mediated by the stimulation of the TRβ1 receptor in the liver by T3 [145]. Therefore, reduction of serum cholesterol by TRβ-selective TH analogs without adverse thyromimetic effects has obvious therapeutic benefits. The TRβ specificity of such analogs would indeed prevent unwanted cardiovascular effects of TH analogs mediated by TRα1. However, it would not prevent the suppression of the hypothalamus-pituitary-thyroid (HPT) axis by such analogs via the TRβ2 receptor expressed in hypothalamus and pituitary. TH receptors are located in the nucleus and the action of T3 and analogs requires their cellular uptake by plasma membrane transporters. This makes it possible to target lipid-lowering TH analogs to the liver through liver-specific transporters.

One of the TH analogs developed to lower cholesterol is eprotirome (KB2115; 3-[[3,5-dibromo-4-[4-hydroxy-3-(1-methylethyl)-phenoxy]-phenyl]-amino]-3-oxopropanoic acid) [146]. It differs from T3 by the presence of two bromines instead of iodine substituents in the inner ring, an isopropyl group instead of an iodine substituent in the outer ring, and a 3-amino-3-oxopropionic acid instead of an alanine side chain (Supplemental Figure 1). It has two characteristics which makes it an attractive candidate as lipid lowering drug: 1) modestly higher affinity for TRβ than for TRα, and 2) minimal non-hepatic tissue uptake. Interestingly, recent rodent studies demonstrated reduction of cholesterol by T3 and eprotirome through a LDL receptor independent pathway [147], indicating a mechanism distinct from other lipid lowering agents. In a 12-week clinical trial where the TH analog was given in addition to statin therapy, eprotirome produced a marked decrease in serum cholesterol levels without adverse extra-hepatic thyromimetic effects [146]. However, phase III clinical trials were discontinued because long-term studies in dogs resulted in cartilage damage [148].

It is important to gain insight in eprotirome's mechanism of action and its pronounced liver-selective uptake for future development of lipid-lowering TH analogs. As mentioned above, TH action is dependent on cellular uptake by plasma membrane transporters, which may also apply to TH analogs. Therefore, the objective of our study was to get a better understanding of the tissue-specific actions of eprotirome by exploring the role of TH transporters in tissue uptake of eprotirome. Because of the presence of a negatively charged side chain in eprotirome, we focused primarily on the possible involvement of organic anion transporters. Different (Na-independent) organic anion transporting polypeptides (OATPs) as well as Na/taurocholate co-transporting polypep-

tide (NTCP; SLC10A1) are capable of transporting TH [20,21,61]. Therefore, we concentrated on the OATP and SLC10 families in our search for transporters facilitating cellular uptake of eprotirome. For comparison, we also tested possible eprotirome transport by MCT8 (SLC16A2) and MCT10 (SLC16A10), the most effective TH transporters identified to date [37].

Materials and methods

Materials

Eprotirome and [^{14}C]eprotirome were obtained from Karo Bio AB (Huddinge, Sweden); iodothyronine derivatives, D-glucose, bovine serum albumin (BSA), Na_2SeO_3, cholate, taurocholate, bromosulfophthalein (BSP), dehydroepiandrosterone 3-sulfate (DHEAS), estrone 3-sulfate (E3S), fetal bovine serum (FBS) from Sigma Aldrich (Zwijndrecht, The Netherlands [NL]); cell culture dishes from Corning (Schiphol, NL); DMEM/F12-GlutaMAX, Dulbecco's phosphate buffered saline (DPBS) and penicillin/streptomycin from Invitrogen (Bleiswijk, NL); X-tremeGENE 9 transfection reagent from Roche (Almere, NL); SYBR Green from Eurogentec (Maastricht, NL); and Na^{125}I from Perkin-Elmer (Groningen, NL). ^{125}I-labeled T3, 3,3′,5-triiodothyroacetic acid (Triac; TA3), 3,3′,5-triiodothyropropionic acid (TP3), T4 and 3,3′,5,5′-tetraiodothyroacetic acid (Tetrac; TA4) were produced as previously described [128].

Plasmids

Human (h) OATP1A2 in pSPORT1 was kindly provided by Prof. Dr. Peter J. Meier (Institute of Clinical Pharmacology and Toxicology, University Hospital Zürich, Zürich, Switzerland), and subcloned into pSG5 (Stratagene, La Jolla, USA). hOATP1B3 in pcDNA3.1-Hygro was kindly provided by Prof. Dr. Dietrich Keppler (German Cancer Research Center, Heidelberg, Germany), and subcloned into pSG5. hOATP1B1 in pCMV6-XL4 [149], hOATP1C1 in pcDNA3.1 [150], hSLC10A1, hSLC10A2 and hSLC10A3 in pcDNA3, rat (r) Slc10a4, hSLC10A5, hSLC10A6, and hSLC10A7 in pcDNA5 [21], hMCT8 in pcDNA3 [24] and hMCT10 in pcDNA3.1 [25] were obtained as described previously. hOATP2A1, and hOATP2B1 in pCMV.SPORT6 were purchased from Open Biosystems (Huntsville, AL). hSLC10A1 and mSlc10a1 were subcloned into pSG5 with a 5′-FLAG tag and an optimized Kozak sequence as previously described [151].

Cell culture and transfection

Most transport assays were done using transfected COS1 (African green monkey kidney) cells but some experiments were also done with JEG3 (human choriocarcinoma) cells. Cells were maintained in culture medium (DMEM/F12+glutamax, 9% FBS, 1% penicillin/

streptomycin, 100 nM Na_2SeO_3) at 37 C and 5% CO_2. Cells were seeded in 6-well or 24-well culture dishes in culture medium without antibiotics. At 70% confluence, cells were transfected with 200 ng (24-well dishes) or 500 ng (6-well dishes) transporter plasmid or empty vector using X-tremeGENE 9 transfection reagent in a 3:1 ratio, according to the manufacturer's protocol.

Transport studies

Time-dependent uptake assays were done in 6-well dishes. All other transport studies were performed in 24-well dishes, producing similar results as in 6-well dishes. Experiments were carried out 48 h after transfection. Cells were rinsed with incubation medium (DPBS/0.1% BSA/0.1% D-glucose or DMEM/F12-GlutaMAX with 0.1% BSA) and incubated for 2-60 minutes at 37 C and 5% CO_2 with 0.5 µM ($6x10^4$ or $12x10^4$ cpm) [^{14}C] eprotirome in 0.5 or 1 ml incubation medium in 24-well or 6-well dishes, respectively. After incubation, cells were rinsed with incubation medium, lysed in 0.5 ml 0.1 M NaOH, mixed with 4 ml scintillation fluid (Pico-Fluor 15, Perkin-Elmer), and counted for radioactivity in a β-counter (Perkin-Elmer). Experiments exploring the kinetics of eprotirome transport, and competition by alternative substrates were carried out in incubation medium without BSA.

To study the Na^+ dependence of [^{14}C]eprotirome transport by SLC10A1, experiments were carried out in Na^+ replete medium (142.9 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 KH_2PO_4, 1.8 mM $CaCl_2$, 20 mM HEPES, 0.1% BSA, pH 7.4) or in Na^+ deplete medium where NaCl was replaced by an equimolar amount of choline chloride.

To study efflux of [^{14}C]eprotirome, cells transfected with SLC10A1 or empty vector were loaded for 30 minutes with [^{14}C]eprotirome in incubation medium. Immediately after removing the loading medium, cells were incubated for 2-30 minutes in efflux medium, consisting of the Na^+ replete or Na^+ deplete medium described above containing 1% BSA to increase the binding of [^{14}C]eprotirome released from the cells. In some experiments, 100 µM cholate was added to the efflux medium to block the SLC10A1 transport channel.

To explore the specificity of eprotirome transport by SLC10A1, transfected COS1 cells were incubated for 30 minutes with 0.5 µM [^{14}C]eprotirome in the absence or presence of 50 µM of the SLC10A1 substrates cholate, taurocholate, BSP, DHEAS or E3S, or the iodothyronine derivatives T4, TA4, T3, TA3, TP3, or 3,5-diiodothyropropionic acid (DP2) (Supplemental Figure 1) in incubation medium without BSA. In addition, possible transport of iodothyronine derivatives by SLC10A1 was tested by incubation of transfected COS1 cells for 30 minutes with 1 nM ($5x10^4$ cpm) [^{125}I]T3, [^{125}I]TA3, [^{125}I]TP3, [^{125}I]T4 or [^{125}I] TA4, and cellular radioactivity was measured as described above using a γ counter [152].

Kinetics of eprotirome transport by SLC10A1 were explored by incubation of transfected COS1 cells for 30 minutes with 0.5 µM [^{14}C]eprotirome and 0.5-200 µM unlabeled

eprotirome in incubation medium without BSA. SLC10A1-mediated uptake was calculated by subtracting uptake in cells transfected with empty vector from uptake in cells transfected with SLC10A1, and expressed as nmol/min. Km values were calculated by Michaelis-Menten analysis using GraphPad Prism 5.01 (GraphPad Software, San Diego CA).

Effect of liver transporters on nuclear activity of eprotirome

To explore the effect of SLC10A1 on the nuclear availability of eprotirome, we used a TRβ1-dependent transactivation assay as described previously with minor modifications [153]. COS1 and JEG3 cells were cultured in 96-well plates, and co-transfected with 15 ng pcDNA3.1-hTRβ1, 15 ng of the pdV-L1 plasmid containing a T3 response element (TRE)-dependent firefly luciferase reporter and a control renilla luciferase reporter [129], and 15 ng pcDNA3-SLC10A1 or empty pcDNA3 vector. Two days after transfection, cells were incubated for 24 hours with 0, 0.1 or 1 nM eprotirome in DMEM/F12-GlutaMAX containing 0.1% BSA. Firefly luciferase and renilla luciferase activities were determined using the Dual-Glo Luciferase Assay (Promega), and the luciferase/renilla ratio was calculated as a read-out for eprotirome transcriptional activity.

Quantitative RT-PCR of tissue SLC10A1 mRNA levels

cDNA prepared from human liver, cartilage, bone marrow, sternum, and chondrosarcoma was kindly provided by Dr. Bram van der Eerden (Erasmus University Medical Center). SLC10A1 qPCR was carried out using 5'-GGTTCTCATTCCTTGCACCA-3' as the forward primer, 5'-ATGGCAGAGAGAACTGTGACG-3' as the reverse primer, SYBRgreen as the probe, and universal master mix (Roche). SLC10A1 expression levels were expressed relative to the expression of the HPRT1 housekeeping gene measured using a commercial primer-probe mix (Applied Biosystems).

Immunoblotting

Expression of FLAG-hSLC10A1 and FLAG-mSlc10a1 protein in COS1 cells was determined by immunoblotting using anti-FLAG antibody as previously described [154]. In brief, transfected COS1 cells were lysed in ice-cold RIPA buffer supplemented with the Complete Protease Inhibitor cocktail (Roche Diagnostics). Lysates were cleared by centrifugation for 5 minutes at 700x*g*, and protein concentration of the supernatant was determined using the bicinchoninic assay (Fisher Scientific). Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes, which were blocked in TBST/5% milk and probed overnight with a 1:1000 dilution of mouse FLAG M2-antibody (F1804; Sigma-Aldrich) at 4 C. Bound antibody was detected with a horseradish peroxidase-conjugated goat anti-mouse antibody (#172-1011; Bio-Rad)

and visualized by enhanced chemiluminescence using the Alliance 4.0 Uvitec platform (Uvitec Ltd).

Eprotirome pharmacokinetics in wild-type and Slc10a1 deficient mice

Male Slc10a1 knockout mice (C57Bl6/J background), and wild-type or heterozygous littermates were housed and bred in the Academic Medical Center, Amsterdam. As heterozygote littermates have almost 90% of normal NTCP expression and indistinguishable from wild-type [155], these were used as control.

To investigate eprotirome transport in control and Slc10a1 knockout mice, the gall bladder was cannulated and bile was collected after distal ligation of the common bile duct, as described [156]. After a 30 minutes depletion-phase, a single bolus of ^{14}C-labeled eprotirome was administered intravenously in 100 µL 0.9% NaCl per 20 g mouse. Blood and bile samples were collected at the indicated time points after eprotirome administration. Mice were sacrificed and liver, kidney, heart and brain were dissolved in Solvable (Perkin Elmer). Radioactivity in plasma and bile was measured by liquid scintillation counting. Total blood volume was estimated based on the mouse body weight (58.5 uL/g mouse).

Statistical analysis

The results are shown as means ± SEM of usually 3 experiments carried out at least in duplicate. GraphPad Prism 5.01 was used for statistical analysis. To test the significance of the difference between background and transporter-mediated eprotirome uptake, a 1-way ANOVA with Bonferroni post-test was used. A paired t-test was used to test the difference in uptake of different substrates between cells transfected with empty vector or with SLC10A1. $P<0.05$ was considered significant.

Results

In addition to the TH transporters MCT8 and MCT10, 6 members of the OATP family and all 7 members of the SLC10 family were tested for [^{14}C]eprotirome uptake in transfected COS1 cells (Figure 1A). Of all 18 transporters tested, SLC10A1 showed a robust increase in eprotirome uptake compared with empty vector. OATP1A2, OATP1B1, OATP1B3, SLC10A6, and SLC10A7 produced a modest, insignificant increase in eprotirome uptake. Figure 1B shows a strong time-dependent increase in eprotirome uptake by COS1 cells transfected with SLC10A1, whereas no increase in eprotirome uptake with time was observed in cells transfected with empty vector. The latter suggests that COS1 cells do not express endogenous transporters for eprotirome. The fold stimulation of eprotirome uptake induced by SLC10A1 *vs.* empty vector increased from 1.4 at 2 minutes to 7.1 at

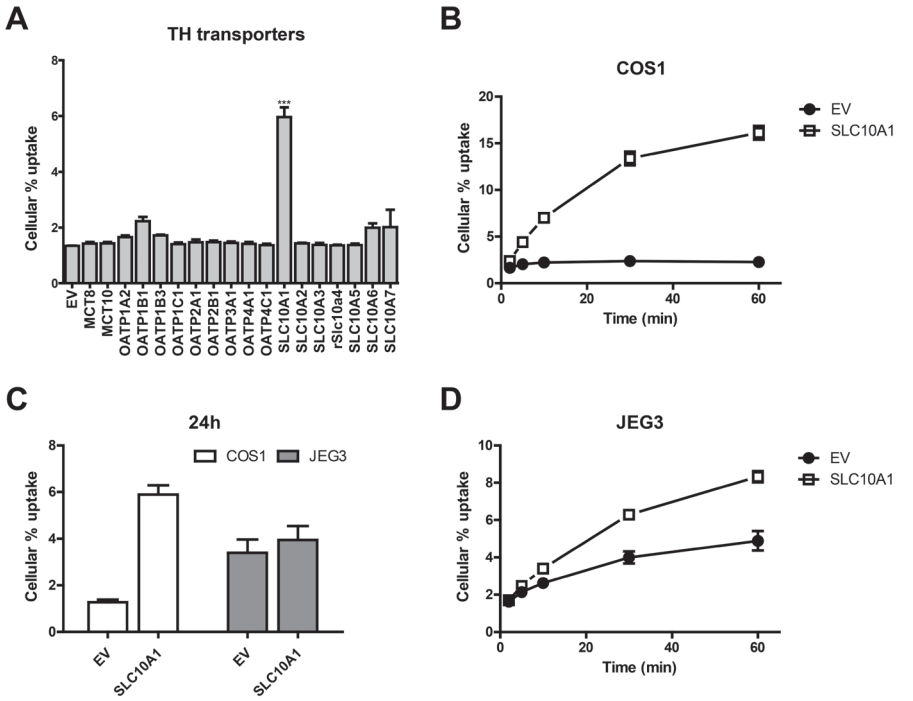


Figure 1. (A) 0.5µM [^{14}C]Eprotirome uptake (30 min) by MCT, OATP and SLC10 transporters in COS1 cells. **(B)** Time-dependent uptake of 0.5µM [^{14}C]eprotirome by COS1 cells transfected with empty vector (EV) or SLC10A1. **(C)** 0.5µM [^{14}C]Eprotirome uptake (24 h) by EV or SLC10A1 transfected COS1 and JEG3 cells. **(D)** Time-dependent uptake of 0.5µM [^{14}C]eprotirome by EV or SLC10A1 transfected JEG3 cells. Results are presented as means ± SEM (n=3). Significance of difference between control and transporter-expressing cells (1-way ANOVA with Bonferroni post-test): * P<0.05; ** P<0.01; *** P<0.001.

60 minutes. Even after 24 hours, a 4.6-fold induction in eprotirome uptake was seen in COS1 cells expressing SLC10A1 (Figure 1C). In contrast to COS1 cells, control JEG3 cells showed a significant time-dependent uptake of eprotirome, representing expression of endogenous transporter(s) that facilitate eprotirome uptake (Figure 1D). Transfection of JEG3 cells with SLC10A1 produced a time-dependent increase in eprotirome uptake up to 1.7-fold at 60 minutes. However, no significant induction of eprotirome uptake by SLC10A1 was found anymore after 24 hours in JEG3 cells (Figure 1C).

SLC10A1 (NTCP) transports its substrates in a Na^+-dependent manner [20,85]. To assess if eprotirome transport by SLC10A1 is also a Na^+-dependent process, parallel incubations were carried out with Na^+ replete medium or with Na^+ deplete medium where all Na^+ was replaced by choline. In both COS1 and JEG3 cells, eprotirome transport by SLC10A1 was found to be strictly dependent on the presence of Na^+ in the medium (Figure 2A). Interestingly, eprotirome uptake by control JEG3 cells was not affected by Na^+ depletion, indicating the involvement of Na^+-independent endogenous transporter(s)

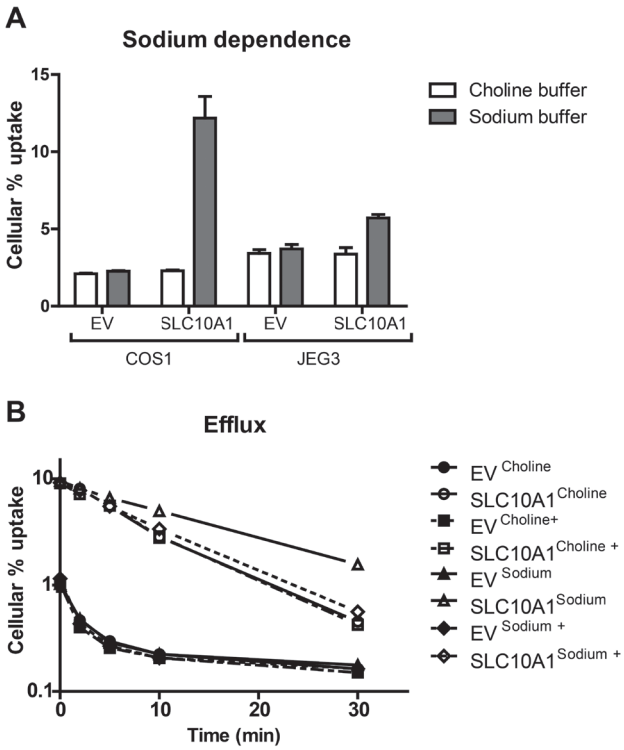


Figure 2. (A) 0.5µM [^{14}C]Eprotirome uptake by control (EV) or SLC10A1-expressing COS1 or JEG3 cells during 30 min incubation in Na^+ replete (sodium) or Na+ deplete (choline) medium. **(B)** Time-dependent efflux of eprotirome from EV or SLC10A1-transfected COS1 cells preloaded during 30 min with 0.5µM [^{14}C]eprotirome. Efflux was studied using Na^+ replete or Na^+ deplete medium containing 1% BSA without or with (+) 100 µM cholate. Results are presented as means ± SEM (n=3).

and thus distinct from SLC10A1. Because of the considerable endogenous uptake by JEG3 cells, further transport characteristics of eprotirome by SLC10A1 were investigated using COS1 cells.

Next, we examined if, in addition to eprotirome uptake, SLC10A1 also facilitates eprotirome efflux. COS1 cells were loaded during incubation for 30 minutes with [^{14}C] eprotirome in incubation medium. Efflux of cellular [^{14}C]eprotirome was subsequently analyzed by incubation with medium containing 1% BSA to minimize re-uptake of [^{14}C]eprotirome released from the cells. Also, the effects of Na^+ depletion from the efflux medium and/or the addition of the SLC10A1 substrate cholate (100 µM) were tested on eprotirome efflux. Apparently, SLC10A1-transfected cells showed the slowest eprotirome efflux in Na^+ replete efflux medium without cholate (Figure 2B). Eprotirome efflux from SLC10A1-transfected cells was similarly increased by Na^+ depletion and by addition of cholate to Na^+ replete medium. Eprotirome efflux from SLC10A1-expressing

cells was not further increased by addition of cholate to Na^+ deplete medium. These data suggest that the presence of 1% BSA in the efflux medium does not completely prevent eprotirome re-uptake, and that this is only accomplished by depletion of Na^+ or addition of cholate. The findings that Na^+ depletion and cholate do not inhibit eprotirome efflux suggest that this is not mediated by SLC10A1, although efflux from cells transfected with empty vector followed different kinetics. The uptake of eprotirome by control cells is much lower than the uptake in SLC10A1-transfected cells and plateaus after a very short incubation time (~10 minutes). It cannot be excluded that a part of the eprotirome is associated with but not internalized by the control cells, which then shows a rapid dissociation from the cells. Na^+ depletion and cholate also did not affect efflux of eprotirome from control cells.

The kinetics of eprotirome transport by SLC10A1 were studied by incubation of transfected cells with 0.5-200 μM eprotirome, and subtraction of eprotirome uptake by control cells from uptake by SLC10A1-expressing cells. The results clearly demonstrate saturation of eprotirome transport by SLC10A1 (Figure 3). Based on these data a Km value of approximately 10 μM was estimated.

We examined if SLC10A1 increases the nuclear availability of eprotirome using COS1 and JEG3 cells co-transfected with TRβ1 and a construct coding for a TRE-dependent luciferase reporter and a control renilla reporter. The luciferase/renilla ratio was used as a measure of the transcriptional activity of eprotirome. In the absence of SLC10A1, 0.1 nM eprotirome was inactive while 1 nM eprotirome induced a modest increase in transcriptional activity (Figure 4A). In contrast, a marked, dose-dependent transcriptional activity was observed in JEG3 cells (Figure 4B). Expression of SLC10A1 resulted in a 5.7- and 4.3-fold increase in transcriptional activity in COS1 cells exposed to 0.1 and 1 nM eprotirome, respectively. In JEG3 cells, SLC10A1 expression enhanced transcriptional activity of 0.1 nM but not of 1 nM eprotirome, where the latter already shows maximum activity in the absence of SLC10A1.

As SLC10A1 also mediates transport of bile acids, steroid sulfates and cholephilic compounds such as BSP [20,21,85], we studied the inhibitory effects of 50 μM taurocholate, cholate, DHEAS, E3S and BSP on eprotirome transport by SLC10A1 (Figure 5A). Eprotirome transport was inhibited in decreasing order of potency by taurocholate ~ cholate > BSP ~ E3S > DHEAS.

The preceding experiments indicate that eprotirome is an excellent substrate for the organic anion transporter SLC10A1. Therefore, we investigated if other TH analogs with a negatively charged side chains could also be substrates for SLC10A1. This was studied indirectly by measuring the effects of TA4, TA3, TP3 and DP2 on eprotirome uptake by SLC10A1 in comparison with the effects of T4 and T3. As expected, minimal effects on eprotirome uptake were seen by addition of 50 μM T3 or T4 (Figure 5B). However, eprotirome uptake was markedly inhibited in decreasing order of potency by 50 μM

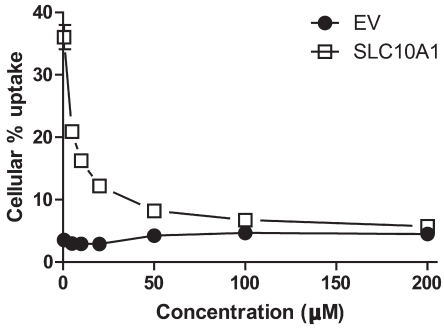


Figure 3. Inhibition of uptake of 0.5 μM [^{14}C]eprotirome in COS1 cells transfected with SLC10A1 or empty vector (EV) by 0.5-200 μM unlabeled eprotirome (30 min incubation). Results are presented as means ± SEM (n=3).

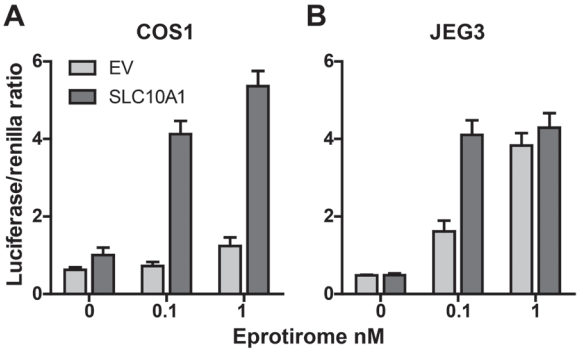


Figure 4. Effects of SLC10A1 on nuclear activity of eprotirome. COS1 **(A)** or JEG3 **(B)** cells co-transfected with TRβ1, TRE-luciferase/control renilla construct, and SLC10A1 or empty vector (EV) were incubated for 24 h with 0, 0.1 or 1 nM eprotirome. The luciferase/renilla ratio was determined as a measure of TRE-mediated transcriptional activity. Results are presented as means ± SEM (n=3).

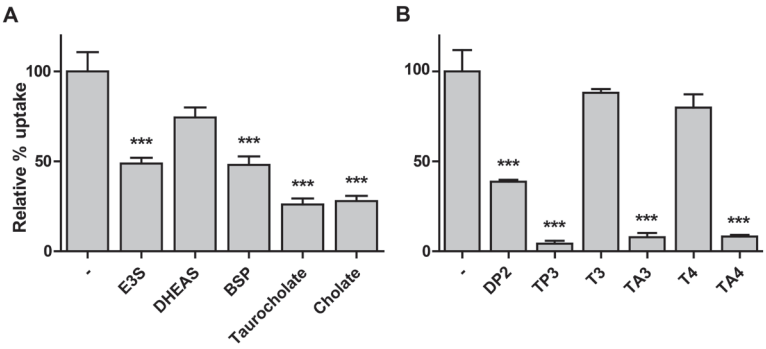


Figure 5. Effects of 50 μM alternative substrates **(A)** and of 50 μM iodothyronine derivatives **(B)** on uptake of 0.5 μM [^{14}C]eprotirome (30 min) by SLC10A1 in COS1 cells. Uptake by SLC10A1-transfected cells is corrected for uptake in control (EV) cells, and expressed relative to uptake in the absence of competitor. Results are presented as means ± SEM (n=3-4). Significance of difference between uptake in the presence *vs.* absence of competitor (1-way ANOVA with Bonferroni post-test): * P<0.05; ** P<0.01; *** P<0.001.

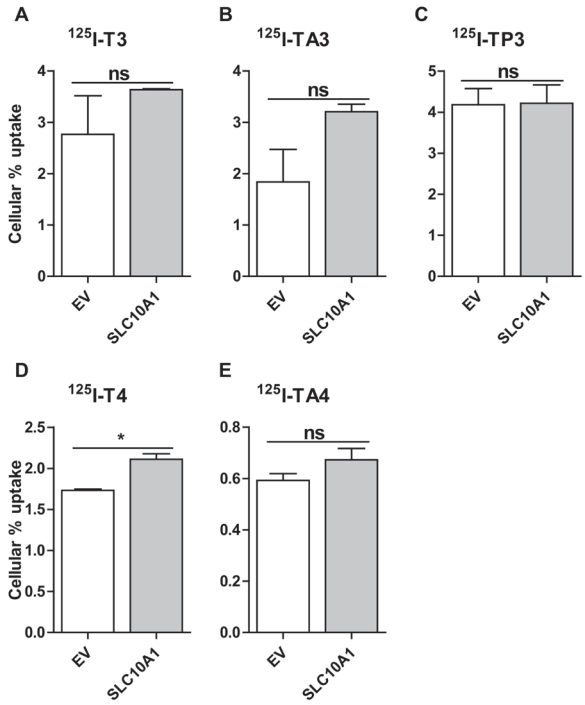


Figure 6. (A-E) Uptake of 1 nM [^{125}I]T3 **(A)**, [^{125}I]TA3 **(B)**, [^{125}I]TP3 **(C)**, [^{125}I]T4 **(D)** and [^{125}I]-TA4 **(E)** during 10 min incubation with EV or SLC10A1 transfected COS1 cells. Results are presented as means ± SEM (n=3). Significance of difference between EV *vs.* SLC10A1 (paired t-test): * P<0.05; ** P<0.01; *** P<0.001.

TP3 > TA3 > TA4 > DP2. Dose-dependent inhibition of eprotirome uptake by SLC10A1 in the presence of increasing concentrations of TA4, TA3, TP3 and DP2 is presented in Supplemental Figure 2, indicating near-complete inhibition at the highest concentration of the analogs tested (100 µM).

To explore if the TH analogs were also substrates for SLC10A1, transport was directly measured in transfected COS1 cells using [^{125}I]T3 (Figure 6A), [^{125}I]TA3 (Figure 6B), [^{125}I]TP3 (Figure 6C), [^{125}I]T4 (Figure 6D) and [^{125}I]TA4 (Figure 6E). Although the TH analogs with acidic side chains strongly inhibit eprotirome uptake by SLC10A1, no significant increase in uptake of the [^{125}I]TH analogs was observed in cells transfected with SLC10A1. In agreement with previous results, SLC10A1 showed minimal transport of T4 and no significant transport of T3.

Because of the cartilage damage observed in dogs during long-term treatment with eprotirome, we investigated if this side effect could be explained by SLC10A1 expression in cartilage. Quantitative RT-PCR of SLC10A1 in human tissues showed pronounced expression in liver but negligible expression in cartilage and cartilage-related tissues (Supplemental Figure 3). These findings indicate that SLC10A1 is not involved in the possible targeting of eprotirome to human cartilage.

Our findings suggest that SLC10A1 is an important transporter for hepatic uptake of eprotirome. To test this hypothesis, we studied the pharmacokinetics of eprotirome in Slc10a1 knockout mice and control littermates [155], assuming that eprotirome is also effectively transported by mouse Slc10a1. To compare the expression and activity of mouse and human SLC10A1, COS1 cells were transfected with FLAG-tagged constructs and tested for protein expression by immunoblotting using anti-FLAG antibody and for eprotirome uptake.

Protein expression levels (Figure 7A) and cellular uptake of taurocholate and eprotirome (Figure 7B) were somewhat lower for FLAG-tagged mouse Slc10a1 than for the human SLC10A1 construct. Surprisingly, plasma clearance and biliary excretion of injected [^{14}C]

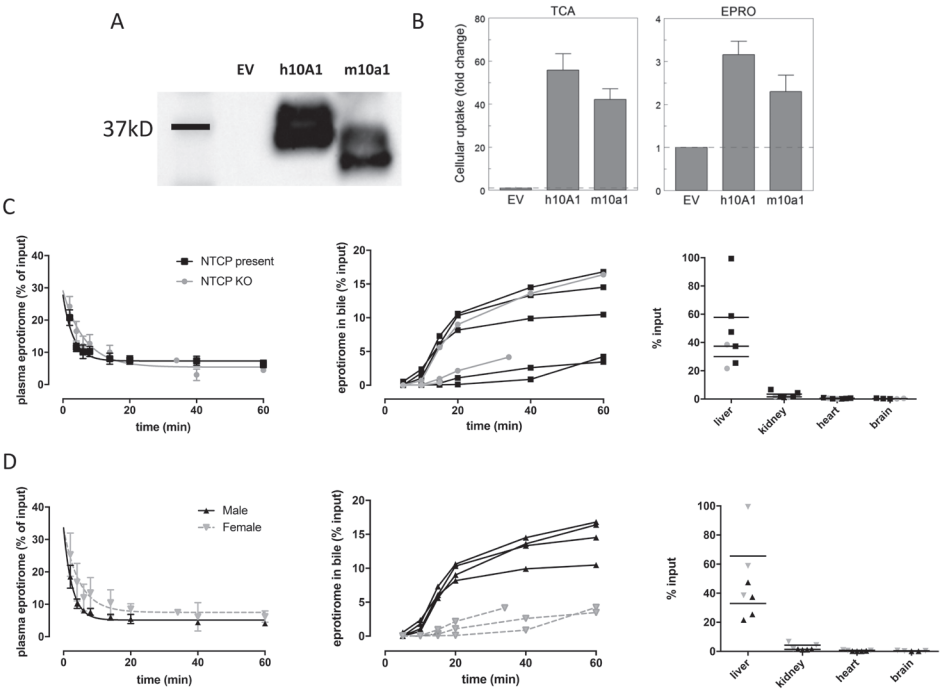


Figure 7. Eprotirome kinetics in NTCP knockout mice and controls. **(A)** Immunoblot of lysates of COS cells transfected with Flag-tagged human SLC10A1 or mouse Slc10a1. **(B)** Uptake of radioactive taurocholate (0.1 μM) or eprotirome (0.5 μM) by COS1 cells transfected with empty vector (EV) or Flag-tagged human SLC10A1 or mouse Slc10a1. Results are presented as means ± SEM (n=3-5). **(C)** Plasma clearance in NTCP knockout mice (grey lines) and control littermates (black lines) was assessed after cannulation of the gall-bladder and jugularis vein. Radiolabeled eprotirome was injected into the jugularis vein and tritium radio-activity was measured in plasma (left figure, data from 4 knockout and 6 control mice) and bile (middle figure, data from 3 knockout and 5 control mice) at the indicated time points, and in tissues at 60 minutes (right figure, data from 2 knockout and 5 control mice). **(D)** Same data as shown in panel C but now separated by sex. Male mice are depicted as black lines and females as grey lines. Plasma data were obtained in 4 males and 5 females (left figure), bile data in 4 males and 4 females (middle figure), and tissue data in 4 males and 3 females.

eprotirome were not significantly different between control and Slc16a1 knockout mice (Figure 7C). However, we noted a strong sex dependence of the data obtained, indicating that both plasma clearance and biliary excretion of eprotirome were markedly higher in male than in female mice irrespective of Slc10a1 status (Figure 7D). The amount of eprotirome present at the end of the experiment was markedly higher in liver than in kidney, heart and brain (Figure 7C,D). However, there was no significant difference between the amounts of eprotirome finally present in the tissues comparing male with female mice or Slc10a1 knockout with control mice.

Discussion

This study shows that transporters are required for transport of eprotirome across the plasma membrane, a necessary step to enable its activation of nuclear T3 receptors. We also demonstrate that eprotirome is effectively transported into cells by the liver-specific transporter SLC10A1. However, the importance of SLC10A1 for the liver targeting of eprotirome in humans remains to be determined.

Since an intact alanine side chain is required for effective transport of iodothyronines by MCT8 and MCT10 [157,158], it is not surprising that eprotirome is not transported by these TH transporters. In view of the negatively charged side chain of eprotirome, we hypothesized that cellular uptake of this TH analog is facilitated by an organic anion transporter. We showed that of the 13 organic anion transporters tested in our first experiments, the bile acid transporter SLC10A1 markedly stimulated cellular uptake of eprotirome. SLC10A1 is known to transport bile acids, steroid sulfates [85] and iodothyronine sulfates [20,21] in a Na^+-dependent manner. This was also found to be the case for eprotirome uptake by SLC10A1, since this was completely blocked under Na^+ deplete conditions. Consistent with previous findings of the unidirectional transport of other substrates [21], SLC10A1 does not appear to mediate cellular efflux of eprotirome.

Experiments using control COS1 cells transfected with empty vector indicate that very little if any eprotirome is taken up by cells through simple diffusion across the plasma membrane. The amount of radioactive eprotirome associated with control COS1 cells does not increase with time after 2 minutes, suggesting that this largely represents binding of eprotirome to the cell surface. This may also explain the rapid release of labeled eprotirome from control COS1 cells in the efflux experiments. The negligible endogenous uptake of eprotirome by COS1 cells permits their use to study the characteristics of eprotirome transport by SLC10A1. Considering the lack of eprotirome transport into control COS1 cells, the 7-fold increase in cell-associated eprotirome after 30 minutes incubation with SLC10A1-transfected cells represents an underestimation of the increase in cellular uptake induced by SLC10A1. These findings indicate that eproti-

rome is effectively transported by SLC10A1, which is further supported by the marked stimulation of the TR-mediated action of eprotirome induced by this transporter. This confirms that cellular uptake of eprotirome by SLC10A1 also results in a prominent increase in the nuclear availability of this T3 analog.

SLC10A1 has a relatively high affinity for eprotirome with an apparent Km value of 7.8 μM, compared with Km values reported for other substrates such as taurocholate (6-34 μM), E3S (27-60 μM) and BSP (3.7 μM) [85]. As expected, transport of eprotirome by SLC10A1 is inhibited by the alternative substrates cholate, taurocholate, E3S, DHEAS and BSP, suggesting that they compete for the same translocation pathway in SLC10A1. The strongest competition was observed with the bile acids cholate and taurocholate.

Obviously, the presence of a negative charge in the substrate is important for its transport by SLC10A1. This is also the case for sulfonated T4 and T3 derivatives (sulfates and sulfamates), which appear to be transported better by SLC10A1 than T4 and T3 themselves [20,21]. The presence of the negatively charged 3-amino-3-oxopropionic acid side chain of eprotirome probably contributes importantly to the effective transport of this T3 analog by SLC10A1. For this reason, we also expected that other iodothyronine derivatives with an anionic side chain, such as TA4, TA3, TP3 and TP2, are also effectively transported by SLC10A1. Although these compounds strongly inhibit eprotirome transport by SLC10A1, studies using ^{125}I-labeled TA4, TA3 and TP3 failed to demonstrate significant transport by SLC10A1. T4 and T3 are poor inhibitors of eprotirome transport by SLC10A1, while they are also poorly transported by SLC10A1. In agreement with previous studies [21], T4 showed minimal uptake by SLC10A1, whereas T3 transport was undetectable. This difference in transport between T4 and T3 may be explained by the presence of a negative charge due to dissociation of the phenolic hydroxyl group of T4 at neutral pH and the absence of such charge on T3. The observation that iodothyronine derivatives with anionic side chains (e.g. TA3, TP3) are potent inhibitors but poor substrates for SLC10A1 in contrast to eprotirome may suggest that the presence of larger iodine *vs.* bromine and isopropyl substituents interferes with the passage of substrate through SLC10A1.

Our studies in mice confirm that eprotirome is largely targeted to the liver. Despite the effective transport of eprotirome by both human and mouse SLC10A1 in transfected cells, its plasma disappearance and biliary clearance were not distinctly different between WT and Slc10a1 KO mice. Instead, a marked influence of sex was observed since both plasma disappearance and biliary clearance of eprotirome were markedly higher in male than in female mice. These differences are most likely explained by a more rapid hepatic uptake of eprotirome in male vs. female mice, although the liver eprotirome content at the end of the experiment was not obviously different between males and females.

Many hepatic genes are expressed sex-dependently in mice, including the liver-specific transporters Oatp1a1 and 1a4. Oatp1a1 is expressed at higher levels in male mice, whereas Oatp1a4 shows higher expression in female mice [159]. The expression of another liver-specific transporter, Oatp1b2, is not clearly sex-dependent [160]. Therefore, Oatp1a1 may also be an important transporter for liver uptake of eprotirome. We were unable to test this hypothesis as we failed to obtain functional expression of Oatp1a1 in transfected mammalian cells. This may be due to the absence of proteins required for expression of Oatp1a1-dependent transport activity, such as Atp11c, Cdc50a, Pdzk1 and perhaps other factors [89,161]. This may also apply to our findings of a low uptake of eprotirome by human OATP1B1 and 1B3, although we previously demonstrated significant transport activity using other substrates [48,149]. We are now exploring optimal conditions for functional expression of OATP1B1 and 1B3, and preliminary results show that this is cell-dependent.

Finally, the lack of an obvious defect in liver eprotirome uptake in Slc10a1 deficient mice may be due to increased expression of other transporters. However, expression of Oatp1a1 and 1b2 is not increased in Slc10a1 KO mice, while expression of Oatp1a4 is only increased in hypercholanemic Slc10a1 KO mice [155].

The small decrease in serum T4 combined with unaffected TSH levels in patients treated with eprotirome suggests some extra-hepatic activity [146,162]. The modest decrease in T4 may be explained by an increase in liver D1 activity, and the lack of an increase in TSH may represent a modest activity of eprotirome at the hypothalamus and/or pituitary [146]. MCT8 is known to be widely expressed in cartilage-forming chondrocytes, while MCT10 appears to be the preferentially expressed transporter in the growth plate [163]. However, these two transporters do not show any eprotirome uptake. The lack of expression of SLC10A1 in human cartilage does not exclude that eprotirome is targeted to cartilage through another transporter, producing the unwanted side effects of eprotirome on this tissue as observed in long-term studies in dogs. Our studies in human cartilage also do not negate that SLC10A1 may be expressed in dog cartilage. More studies are required to resolve this issue.

Finally, clinical studies with eprotirome indicate good oral availability of this T3 analog [146], indicating the presence of transporters for eprotirome in the intestinal mucosa. Our studies demonstrate that the SLC10A2 intestinal bile acid transporter is not involved in the absorption of oral eprotirome.

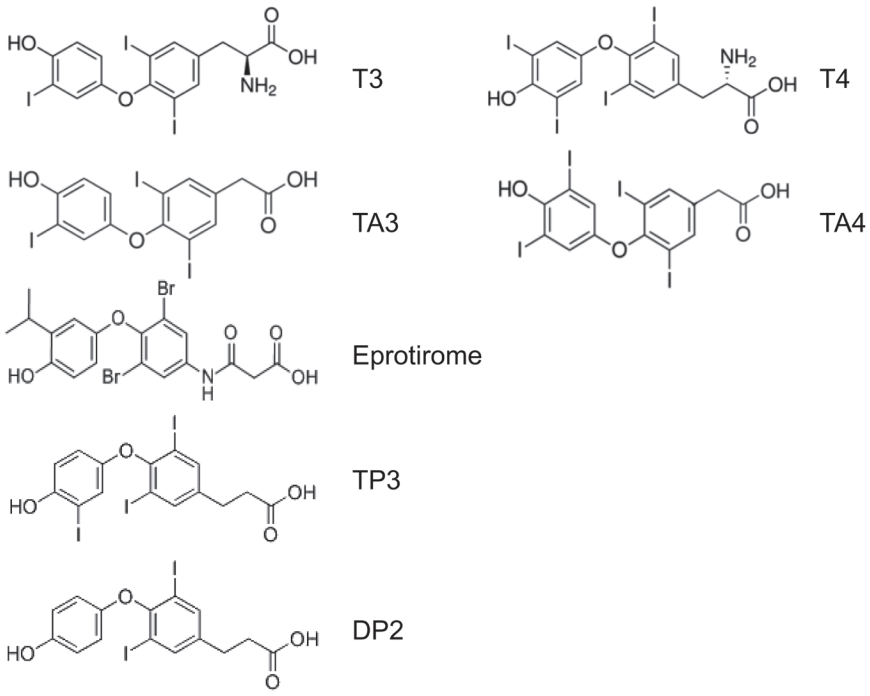
SLC10A1 transports a broad range of substrates, in particular bile acids [85,164], that may interfere with hepatic eprotirome uptake *in vivo*. In addition to physiological substrates, certain drugs may also be transported by SLC10A1, and thus also interfere with eprotirome uptake. Recent studies have indicated that uptake of statins into the liver is partly mediated by SLC10A1 [165-168]. This is of particular interest since statins and eprotirome have the same therapeutic goal, that is, reduction of serum cholesterol. A recent clinical

trial has indicated that the combined therapy with statins and eprotirome produces a greater reduction in serum cholesterol than treatment with statins alone [146]. Despite this positive clinical outcome, our results suggest that the effectiveness of combined TH analog and statin therapy may be limited by drug-drug interaction at the level of hepatic transport.

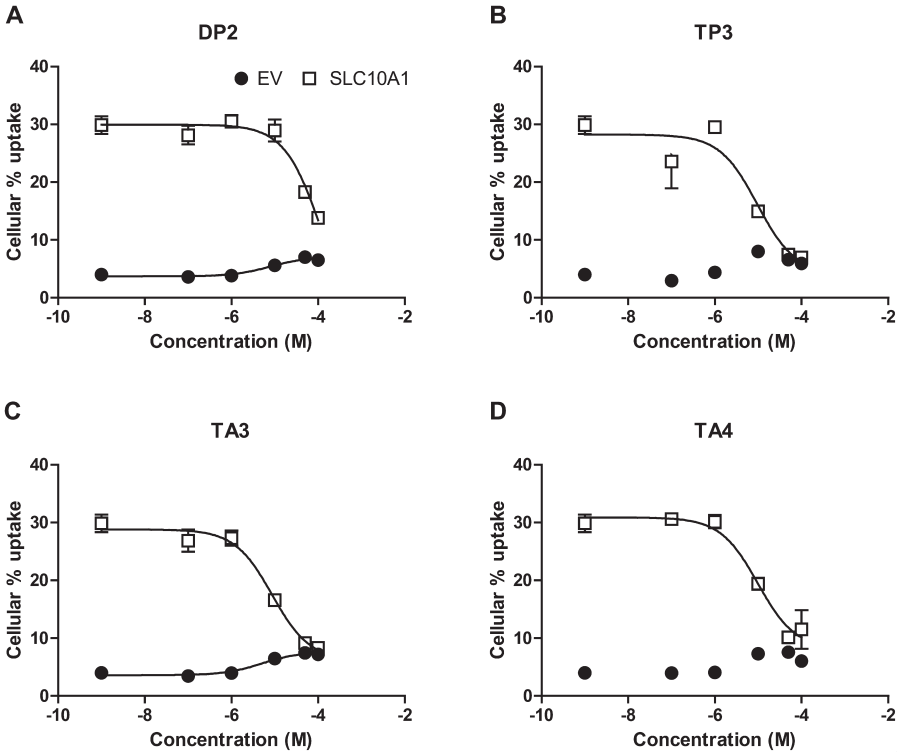
In conclusion, the liver-specific bile acid transporter SLC10A1 effectively transports eprotirome into cells. The high expression of this transporter in liver may explain the liver-selective action of this TH analog. However, hepatic eprotirome uptake is not affected in Slc10a1 knockout mice, suggesting an important contribution of other liver-specific transporters. Our findings support the hypothesis that TH analogs may be targeted to specific tissues depending on the transporters they express.

Acknowledgements

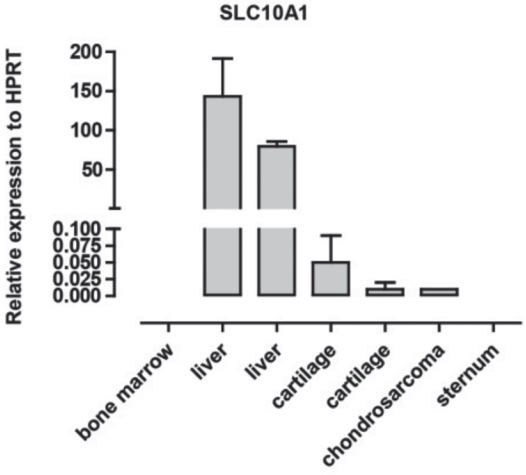
We would like to thank Jane van Heteren for technical assistance, the Erasmus MC tissue bank for providing the human tissues, Dr. Bram van der Eerden for providing cDNA of the tissues tested, and Dr. Peter Meier and Dr. Dietrich Keppler for providing transporter plasmids. Stan FJ van de Graaf is supported by the Netherlands Organization for Scientific Research (Vidi; 91713319).



Supplemental Figure 1. Chemical structures of T3, T4 and analogs.



Supplemental Figure 2. Effects of 0.1-100 µM DP2 **(A)**, TP3 **(B)**, TA3 **(C)** or TA4 **(D)** on uptake of 0.5 µM [^{14}C]eprotirome during 30 min incubation with COS1 cells transfected with SLC10A1 or empty vector (EV). Results are presented as means ± SEM (n=3).



Supplemental Figure 3. Expression of SLC10A1 relative to the HPRT1 housekeeping gene in different tissues. Results are presented as means ± SEM (n=2). For liver and cartilage the results of two different samples are shown.

TRANSPORT OF THE THYROID HORMONE ANALOG GC-1 BY LIVER-SPECIFIC TRANSPORTERS

4

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To be submitted

Abstract

Sobretirome (GC-1) is a thyroid hormone (TH) analog that was developed in the 1990s to lower cholesterol levels through selective binding to the thyroid hormone receptor TRβ1 and preferential accumulation in the liver. GC-1 has been tested in several animal models and clinical trials, all showing significant reduction of serum lipid levels without unwanted thyrotoxic side effects.

We studied the cellular transmembrane transport of GC-1 by the Na-independent organic anion transporters OATP1A2, 1B1, 1B3 and the Na-dependent organic anion transporter SLC10A1 (also known as Na/taurocholate co-transporting polypeptide, NTCP). Cellular transport was studied in transfected COS1 and HEK293 cells using [^{3}H] GC-1, [^{3}H]taurocholate (TCA), and [^{125}I]TH analogs. In addition, we explored the effect of the transporters on the nuclear availability of GC-1 and other analogs, using a TRβ1-dependent transactivation assay.

In transfected HEK293 cells, uptake of GC1 was stimulated 3.8-fold by OATP1B1, 2.0-fold by OATP1B3 and 1.8-fold by NTCP, but not by OATP1A2. Also the uptake of eprotirome was stimulated by NTCP, OATP1B1 and OATP1B3, whereas no significant eprotirome transport was seen with OATP1A2. NTCP was clearly most effective in facilitating transport of TCA. Regarding TH transport, T3 uptake was higher than T4 uptake, and the percentage uptake of both T4 and T3 was higher at 1 nM than at 10 μM substrate concentration for all transporters. In our hands, co-transfection of PDZK1 (a 70 kDa protein with 4 PDZ-domains, necessary for proper expression of OATPs at the plasma membrane) or the cytoplasmic TH binding protein CRYM had little or no effect on T3 and T4 transport. Transfection of OATP1B1 and NTCP increased transcriptional activity in HEK293 cells incubated with 1 nM GC-1, while OATP1A2 showed no stimulation. With eprotirome incubation, OATP1B3 also stimulated transcriptional activity in HEK293 cells.

In conclusion, we have demonstrated that liver-specific transporters OATP1B1, 1B3, and NTCP can transport the TH analog GC-1 across the plasma membrane, a crucial step to enable its activation of nuclear TRβ1 receptor. However, other transporters for both GC-1 and eprotirome seem to be present in liver, which should be further elucidated before TH analogs can safely be targeted to specific tissues.

Introduction

Elevated serum cholesterol and/or triglyceride levels trigger the onset of atherosclerosis, ischemic heart disease, stroke and peripheral vascular abnormalities leading to high cardiovascular mortality in developed countries (http://www.who.int/healthinfo/global_burden_disease/projections/en/). Treatment with statins has achieved striking cholesterol reducing effects; however, there remains a need for new cholesterol-lowering drugs.[169] In the past years, much research has been done focusing on thyroid hormone (TH) signaling with the aim of developing new cholesterol-lowering therapies, since it is known that TH plays an important role in lipid metabolism.

Increased TH levels result in both positive and negative outcomes. On the negative side, hyperthyroidism can cause tachycardia, arrhythmias, bone and muscle catabolism, and mood disturbances. However, reduction of serum cholesterol levels and body fat can be considered as beneficial effects, particularly in patients with dyslipidemia and metabolic syndrome.[145,170] The effects of T3 on the heart, muscle and bone are due to the stimulation of the predominant TH receptor isoform TRα1 in these tissues, while the lipid-lowering effects are mediated by TRβ1 in the liver.[105]

From this perspective, TRβ-selective ligands without undesirable effects via TRα are of great interest for the therapeutic control of serum cholesterol. However, analogs targeting TRβ will suppress the hypothalamus-pituitary-thyroid (HPT) axis, since TRβ2 is highly expressed in hypothalamus and pituitary. Therefore, lipid-lowering TH analogs should ideally be directed to the liver only. TRs are located in the nucleus and the action of T3 and analogs requires their cellular uptake by plasma membrane transporters. This makes it possible to target lipid-lowering TH analogs to the liver through liver-specific transporters without unwanted effects on the HPT-axis or other TRβ-dominant tissues.

In a 12-week clinical trial in patients with hypercholesterolemia, where the TH analog eprotirome (KB2115) was given in addition to statin therapy, eprotirome produced a marked decrease in atherogenic lipoproteins without adverse extra-hepatic thyromimetic effects.[146]

In our previous work,[171] we explored the tissue-selectivity of eprotirome by investigating its transport across the plasma membrane. Our study showed that in particular expression of the liver-specific bile acid transporter Na-taurocholate co-transporting polypeptide (NTCP, SLC10A1) stimulates cellular eprotirome uptake as well as TRβ1-mediated transcriptional activity.

In the current work, we extended our analyses by studying cellular transmembrane transport of another TH agonist, sobetirome (GC-1). Because of the presence of a negatively charged side chain in GC-1, we studied its possible transport by NTCP as well as the organic anion transporting polypeptides OATP1A2, 1B1 and 1B3. Of the latter, OATP1B1 and 1B3 are also selectively expressed in liver.

GC-1 was synthesized in the 1990s and differs structurally from T3 by having methyl and isopropyl substituents instead of the three iodines, a methylene instead of a biaryl ether linkage, and an oxyacetic acid side chain instead of the alanine side chain. GC-1 is reported to preferentially bind TRβ1 with a high affinity and to accumulate selectively in liver.[170,172]

GC-1 has been tested in several animal models including mice, rats and monkeys, and reached Phase 1b human clinical trials. In all models, GC-1 treatment resulted in significantly reduced LDL cholesterol levels and in primates additional reduction of serum triglycerides and lipoprotein a was observed. The used GC-1 doses in these studies did not elicit unwanted thyrotoxic side effects on heart rate and muscle and bone catabolism, except for a weak suppression of the HPT-axis.[173–175]

Unfortunately, long-term eprotirome treatment led to unexpected cartilage defects in dogs after which phase III clinical trials with eprotirome were discontinued (Karo Bio. Karo Bio terminates the eprotirome program. [Press release]. www.karobio.com; 2012 [14 February 2012]. Available from: http://www.karobio.com/investormedia/pressreleaser/pressrelease?pid = 639535). It is important to clarify the occurrence of these harmful effects before future clinical trials with lipid-lowering TH agonists can be considered. In addition, it is necessary to gain more insight in the mechanisms of tissue-specific targeting of TH analogs, such as GC-1, and their interactions with different transporters.

Materials and methods

Materials

GC-1 and [^{3}H]GC-1 were prepared as described.[172] Iodothyronines, bovine serum albumin (BSA), Na_2SeO_3, [^{3}H]taurocholate (TCA), and fetal bovine serum (FBS) were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands [NL]); cell culture dishes from Corning (Schiphol, NL); DMEM/F12-GlutaMAX, Dulbecco's phosphate buffered saline (DPBS) and penicillin/streptomycin from Invitrogen (Bleiswijk, NL); X-tremeGENE 9 transfection reagent from Roche (Almere, NL); and Na^{125}I from Perkin-Elmer (Groningen, NL).^{125}I-labeled T3, T4 and were produced as previously described.[128]

Plasmids

Human OATP1A2, OATP1B1, OATP1B3 and NTCP cDNA were all subcloned in the pSG5 expression vector with an optimized Kozak translation start site and an N-terminal FLAG-tag as previously described.[151,171] In addition, we used an N-terminal FLAG-tagged TRβ1 construct in pcDNA3, and a pdV-L1 reporter construct, expressing firefly luciferase (LUC) under control of a T3 responsive promoter and renilla luciferase (REN) under control of a constitutive promoter as described.[129,176] The constructs for human cytoplasmic

T3 binding protein μ-crystallin (CRYM)[25] and for PDZK1[89,177] were obtained as previously reported.

Cell culture and transfection

COS1 (African green monkey kidney) and HEK293 (human embryonic kidney) cells were used in the experiments, as these cell lines show relatively high transfection efficiency. Cells were maintained in culture medium (DMEM/F12 + glutamax, 9% FBS, 1% penicillin/streptomycin, 100 nM Na_2SeO_3) at 37 C and 5% CO_2. Cells were seeded in 24-well (transport and luciferase studies) or 6-well (immunoblotting) culture dishes in culture medium without antibiotics 24 hours before transfection. At 70% confluence, the medium was changed to DMEM/F12 containing charcoal-stripped FBS and the cells were transiently transfected with 200 ng (24-well) or 500 ng (6-well) plasmid in 0.5 ml (24-well) or 2 ml (6-well). Transfection was performed using X-tremeGENE 9 transfection reagent in a 3:1 ratio, according to the manufacturer's protocol.

Transport studies

Transport studies were performed in cells transfected with 200 ng of transporter plasmid (Flag-OATP1A2, 1B1 or 1B3 or Flag-NTCP) or empty pSG5 vector (EV) with or without addition of 100 ng CRYM or PDZK1 plasmid. Two days after transfection, cells were washed with DMEM/F12 + 0.1% BSA and incubated for 30 minutes at 37 C and 5% CO_2 with 1 nM or 10 μM T3 or T4 and 50,000 cpm [^{125}I]T3 or [^{125}I]T4, or 0.5 μM [^{3}H]GC-1, or 1 μM [^{3}H]TCA in 0.5 ml incubation medium (DMEM/F12 + 0.1% BSA). After incubation, cells were washed with incubation medium, lysed in 0.5 ml 0.1 NaOH, and counted for radioactivity in a γ-counter ([^{125}I]T3 or [^{125}I]T4) or mixed with 4 ml scintillation fluid (Pico-Fluor 15, Perkin-Elmer), and counted for radioactivity in a β-counter ([^{3}H]GC-1 or [^{3}H]TCA). In some experiments, uptake studies were also performed done using DMEM/F12 without BSA.

Luciferase activity

To study the effect of the transporters on the nuclear availability of GC-1 and eprotirome, we used a TRβ1-dependent transactivation assay. COS1 and HEK293 cells were cultured in 24-well dishes and co-transfected with 60 ng transporter plasmid (Flag-OATP1A2, 1B1 or 1B3 or Flag-NTCP) or empty pSG5 vector together with 60 ng pdV-L1 reporter construct and 60 ng Flag-hTRβ. Two days after transfection, cells were incubated for 24 hours without or with 1 nM GC-1 or eprotirome in DMEM/F12 + 0.1% BSA. Firefly luciferase and renilla luciferase activities were determined as previously described,[178] and the LUC/REN ratio was calculated as a read-out for transcriptional activity.

Immunoblotting

The expression of the FLAG-tagged transporters OATP1A2, 1B1, 1B3 and NTCP was determined by immunoblotting using an anti-FLAG antibody as previously reported.[179]

Statistical analysis

All experiments were performed in triplicate and are presented as ± SEM of usually 3 experiments. Statistical differences were calculated using Student's t-test or 2-way ANOVA with Bonferroni post-hoc test. $P < 0.05$ was considered significant.

Results

Initial experiments were performed to compare the efficacy of transporter expression between COS1 and HEK293 cells. To this end, cells transfected with the Flag-tagged OATP and NTCP constructs were analyzed for transporter protein expression by immunoblotting and for functional expression by determining the uptake of the bile acid TCA (Figure 1). Expression of the OATP1A2 protein was clearly less than the expression of the OATP1B1 and 1B3 proteins in both COS1 and HEK293 cells. Surprisingly, NTCP protein could only be visualized in COS1 cells (Figure 1A). Among the different transporters tested, NTCP was clearly most effective in facilitating transport of TCA. Compared with control empty vector-transfected cells, TCA uptake was increased 13.2-fold in COS1 cells and 48.5-fold in HEK293 cells. Modest stimulation of TCA uptake was observed with OATP1B1 and OATP1B3, which again was greater in HEK293 than in COS1 cells. Stimulation of TCA uptake by OATP1A2 was negligible in both COS1 and HEK293 cells (Figure 1B). However, significant stimulation of estrone-3-sulfate (E3S) uptake was observed with all three OATPs, declining in the order OATP1B1 >> 1A2 > 1B3 (Figure 1C). Based on the functional activities of the different transporters in the two cell systems, all further experiments were done using HEK293 cells.

Previous studies have indicated that OATP1A2, 1B1 and 1B3 and NTCP are capable of transport TH. Therefore, we studied T3 and T4 uptake by the Flag-tagged OATPs and NTCP in HEK293 cells, and this was done using a low (1 nM) or a high (10 μM) substrate concentration (Figure 2). In general, T3 uptake was higher than T4 uptake, which may be related to the higher affinity of T4 than of T3 for the BSA (0.1%) present in the medium. Also in general, the percentage uptake of both T4 and T3 was higher at 1 nM than at 10 μM substrate concentration, suggesting saturation of the different endogenous and exogenous transporters. Apparently, endogenous transporters are saturated by 10 μM substrate to a greater extent than the exogenous transporters, resulting in a greater fold induction of TH uptake by transfected transporters compared with 1 nM substrate. At the high substrate concentration, uptake of T3 and T4 was stimulated 1.6- and 1.2-fold

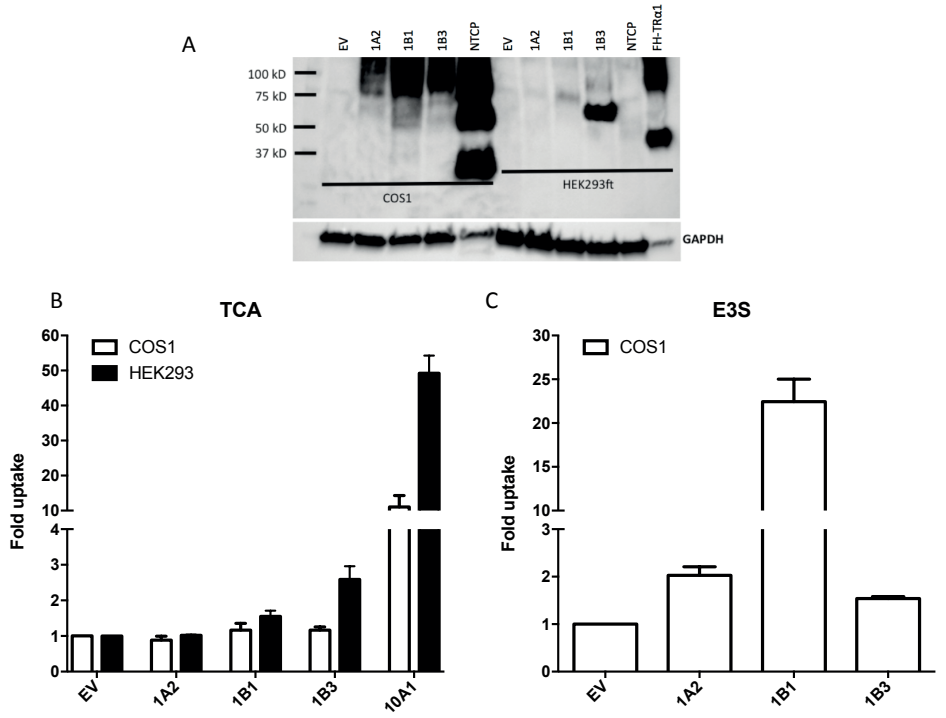

Figure 1. (A) Immunoblot of lysates of COS1 and HEK293 cells transfected with Flag-tagged OATPs and NTCP. Flag/HA-tagged TRα1 was used as a positive control.
Uptake of radioactive taurocholate **(B)** and estrone-3-sulfate **(C)** by COS1 and HEK293 cells transfected with empty vector (EV) or Flag-tagged OATPs and NTCP. Results are presented as means ± SEM (n = 3). Highest stimulation of TCA uptake was observed with NTCP, while OATP1B1 showed the most effective E3S transport.

by OATP1A2, 3.5- and 2.6-fold by OATP1B1, 2.3- and 3.1-fold by OATP1B3, and 2.4- and 2.7-fold by NTCP, respectively.

Since it has been reported that different OATPs require interaction with PDZK1, a 70 kDa protein with 4 PDZ-domains,[87–89] for proper expression at the plasma membrane, we have explored the effect of co-transfection of PDZK1 with OATP1A2, 1B1 or 1B3. In our hands, co-transfection of PDZK1 had little or no effect on T3 and T4 transport by OATP1A1, 1B1 and 1B3 (not shown)

In addition, it has been shown previously that expression of the cytoplasmic TH binding protein CRYM augments the cellular accumulation of T3 and T4 induced by the TH transporters MCT8 and MCT10.[137] This is explained by the property that MCT8 and MCT10 facilitate both cellular uptake and efflux of iodothyronines. To investigate if this also applies to OATP1A2, 1B1, 1B3, and NTCP, we tested the effect of co-transfection of CRYM with these transporters on T3 and T4 uptake. CRYM was found to have little or

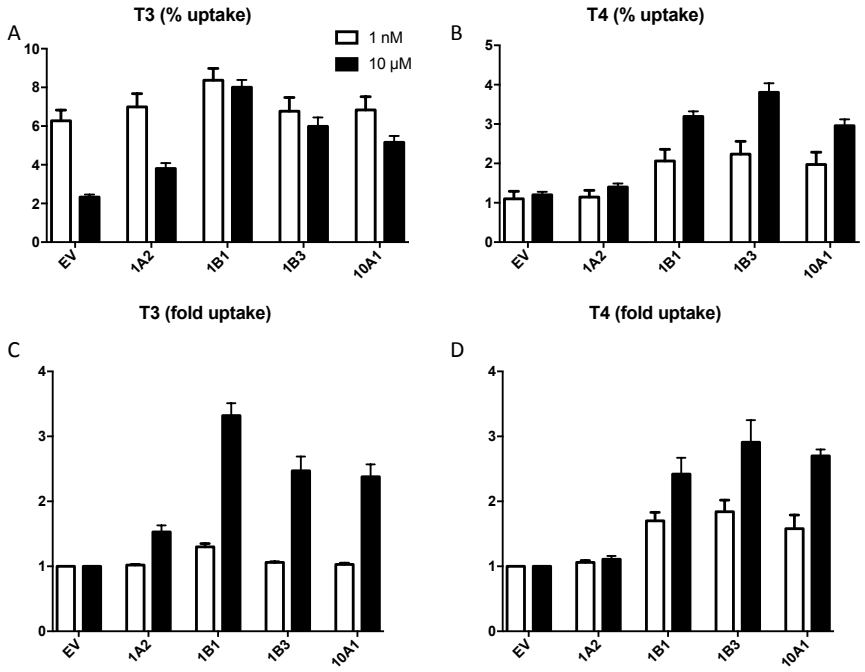

Figure 2. Uptake of radioactive T3 and T4 (1 nM vs 10 µM) by HEK293 cells transfected with empty vector (EV) or Flag-tagged OATPs and NTCP in medium containing 0.1% BSA. The highest uptake by endogenous transporters was seen for 1 nM T3. With 10 µM T3 transfected OATP1B1 showed the greatest fold induction of TH uptake, while for 10 µM T4 transfected OATP1B3 stimulated the uptake most effectively.

no effect on the cellular accumulation of T4 and T3 when incubated at low (1 nM) or high (10 µM) substrate concentration with cells transfected with empty vector or the different transporters (not shown). These findings suggest that OATP1A2, 1B1, 1B3 and NTCP primarily facilitate the uptake rather than the efflux of TH.

In a previous study using transfected COS1 cells, we have demonstrated effective transport of eprotirome by NTCP and modest transport by OATP1B1, whereas OATP1A2 and OATP1B3 appeared to be inactive. However, these studies were done with untagged constructs and the level of transporter expression was unknown. Therefore, we decided to study the transport of eprotirome in HEK293 cells transfected with the Flag-tagged OATP and NTCP constructs. The results indicated that uptake of eprotirome is stimulated not only by NTCP and OATP1B1 but also by OATP1B3, whereas still no significant eprotirome transport was seen with OATP1A2 (Figure 3A).

We also examined the transport of the other liver-selective T3 analog GC-1 by the different Flag-tagged transporters in transfected HEK293 cells. Compared with control empty vector-transfected cells, uptake of GC1 was stimulated 3.8-fold by OATP1B1, 2.0-fold by OATP1B3 and 1.8-fold by NTCP, but not by OATP1A2 (Figure 3B).

Next, we examined to what extent OATP1A2, 1B1, 1B3 and NTCP increase the nuclear availability of eprotirome and GC-1 using HEK293 cells co-transfected with Flag-TRβ1 and pdV-L1, a construct coding for a TRE-dependent luciferase reporter and a control renilla reporter. The luciferase/renilla ratio was used as a measure of the transcriptional activity of eprotirome and GC-1.

Incubation of control HEK293 cells with 1 nM eprotirome resulted in a 2.8-fold increase in transcriptional activity, which was further stimulated 2.5-fold by expression of OATP1B1, 1.8-fold by OATP1B3 and 1.9-fold by NTCP but not by OATP1A2 (Figure 3C). Incubation of control cells with 1 nM GC-1 resulted in a 3.1-fold increase in transcriptional activity, which was further stimulated 1.8-fold by OATP1B1 and 1.2-fold by NTCP but not by OATP1A2 or OATP1B3 (Figure 3D).

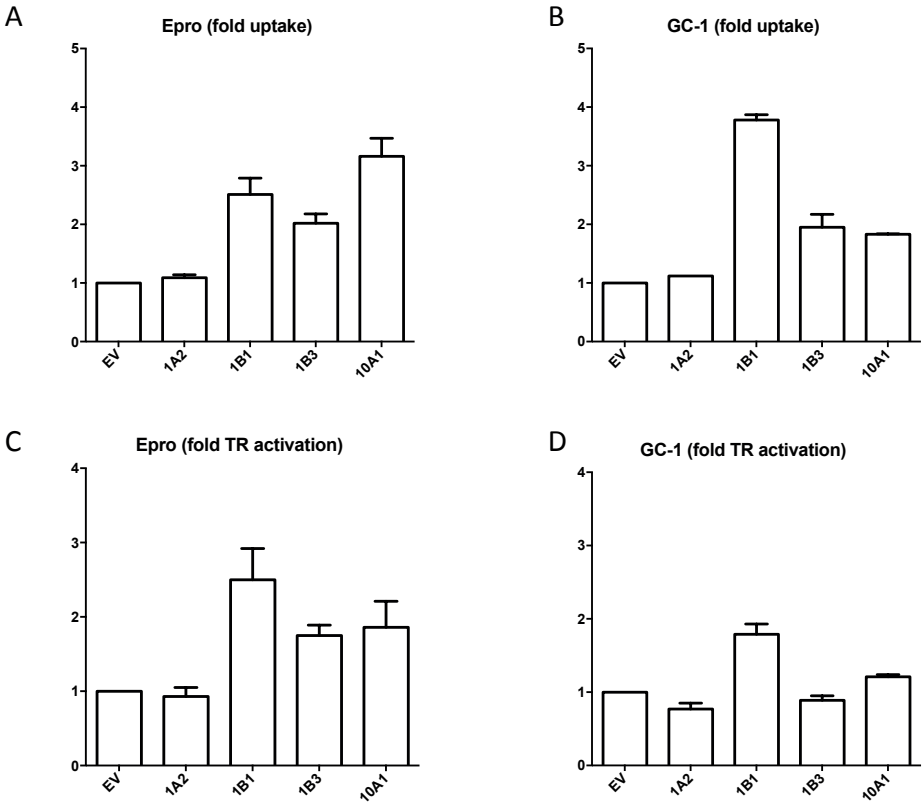

Figure 3. Uptake of radioactive eprotirome **(A)** and GC-1 **(B)** by HEK293 cells transfected with empty vector (EV) or Flag-tagged OATPs and NTCP. Highest stimulation of eprotirome uptake was seen by NTCP, while OATP1B1 facilitated GC-1 uptake most effectively. OATP1A2 did not show eprotirome or GC-1 uptake. Expression of OATP1B1 stimulated transcriptional activity the most in HEK293 cells, incubated with 1 nM eprotirome and GC-1.

Discussion

In this study, we demonstrate that liver-specific transporters OATP1B1, 1B3, 1A2 and NTCP are capable of transporting the TH analog GC-1 across the plasma membrane, a crucial step to enable its activation of nuclear TRβ1 receptor. This supports the hypothesis that transporters may be used to target TH analogs to specific tissues.

In our previous study, we showed that mainly OATP1B1, 1B3 and NTCP facilitate the cellular uptake of the TH agonist eprotirome. Because GC-1 showed similar lipid-lowering effects as eprotirome in both animal studies and human clinical trials[146,162,170] and high structure similarity to eprotirome, we extended our experiments by testing the same transporters in their uptake of GC-1. Besides their ability to facilitate the cellular uptake of GC-1, the transporters OATP1B1, 1B3, 1A2 and NTCP also induced the transport of (mainly high concentrations) T3 and T4 into the cells.

OATP1B1 and OATP1B3 are considered to be liver-specific and are localized at the sinusoidal membrane of hepatocytes where they play an important role in the hepatic clearance of albumin-bound amphipathic organic compounds and also mediate sodium-independent transport of TH, steroids, drugs and bile salts. The expression of OATP1A2 in humans includes the liver but also the brain, kidney and intestine.[48,53,54,180,181] NTCP is expressed at the basolateral membrane of hepatocytes, where it transports bile acids, steroid sulfates and iodothyronine sulfates in a sodium-dependent manner.[20,21,85]

In experiments using COS1 cells transfected with empty vector, very little GC-1 was taken up by the cells suggesting a negligible presence of endogenous transporters for GC-1 in this cell line. In HEK293 cells, the endogenous expression of OATP1A2, 1B1, 1B3, and NTCP is reported to be low[182] however, more endogenous uptake of GC-1 was observed in these cells compared to COS1 cells. This points towards the presence of other, yet unidentified, transporters for GC-1 in HEK293 cells. This is confirmed by a marked stimulation of the TRβ1-mediated action of GC-1 in control HEK293 cells. In contrast, expression of the transporter constructs in COS1 cells effectively increased the nuclear availability of GC-1, confirming cellular GC-1 uptake by these transporters. In addition, in our previous study[171] we showed cellular uptake of eprotirome in HEK293 and JEG3 cells transfected with empty vector. This supports the existence of other transporters for both GC-1 and eprotirome in liver and other tissues. Possible expression of such transporters in cartilage could explain the unwanted side effects of eprotirome in long-term studies in dogs.

Since it has been reported that the tested transporters facilitate the transport of bile acids, we tested the uptake of TCA by these transporters in both COS1 and HEK293 cells. Expression of OATP1B3 and 1B1 resulted in mild cellular uptake of TCA, especially in HEK293 cells, while NTCP showed much more convincing TCA transport into the cells (13.2 vs 48.5 fold in COS1 vs HEK293 cells). Despite the pronounced functional activity

of NTCP in HEK293 cells, we were unable to visualize the expression of the NTCP protein in this cell line by immunoblotting. A clear expression of NTCP in COS1 cells, supports the proper function of our NTCP transporter plasmid and suggests that HEK293 cell-specific factors could interfere with the stability of the used Flag-epitope attached to the NTCP transporter. Possibly, Flag-NTCP undergoes sulfation or ubiquitin-mediated degradation in HEK293 cells, which enables visualization of the protein on Western blot.[183,184] We tested inhibition of Flag sulfation using sodium chlorate and inhibition of ubiquitin-mediated degradation using the proteasome inhibitor MG132 and bortezomib however, this did not improve the expression of Flag-NTCP on immunoblot nor the uptake of TCA by NTCP (data not shown).

In addition, we explored the effect of co-transfecting the ancillary protein PDZK1 since it has been shown that interaction with PDZK1 is necessary for functional expression of many members of the OATP transporters at the plasma membrane.[87–89] Unfortunately, this did not change the uptake capacity nor the protein expression of the tested transporters suggesting that endogenous levels of PDZK1 are sufficient for proper function of the transfected transporter proteins or that other, likely liver-specific factors necessary for OATP-PDZK1 interaction, were lacking in our used cell models. We studied GC-1 transport *in vitro* under conditions that may not represent the *in vivo* situation. Thereby, it is likely that *in vivo* OATP1A2, 1B1, 1B3 and NTCP transport other physiological substrates or drugs, that can interfere with GC-1 transport.

It has been shown in animal studies that TH analogs can lower cholesterol via induction of the LDL-receptor, which promotes cholesterol reabsorption, and CYP7A1, which catalyzes the main step of cholesterol to bile acid conversion. These mechanisms could work additively with statins, which also increase liver LDL-receptor expression and additionally suppress liver HMG-CoA reductase activity.[185–187] From this perspective, it is very intriguing to study liver-specific targeting of cholesterol reducing thyromimetics. However, it is necessary to fully understand whether these TH analogs are exclusively transported to the liver and if these analogs only show TRβ1 selectivity. Our current study unraveled a small piece of the large puzzle regarding the mechanisms of how TH analogs can be targeted to specific tissues depending on the transporters they express.

RESISTANCE TO THYROID HORMONE IN AN 18 MONTHS OLD GIRL DUE TO A MUTATION IN THE THYROID HORMONE RECEPTOR ALPHA 1 AND 2: CLINICAL PHENOTYPE AND THE EFFECT OF TREATMENT

5

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Thyroid 2016

Abstract

Background

Recently, the first patients with resistance to thyroid hormone (RTHα) due to inactivating mutations in the thyroid hormone receptor alpha (TRα) were identified. These patients are characterized by growth retardation, variable motor and cognitive defects, macrocephaly and abnormal thyroid function tests. Our objective was to characterize a young girl (18 months) with a mutation in both TRα1 and TRα2, and study the effects of early levothyroxine (LT4) treatment.

Methods

The patient was assessed clinically and biochemically before and during 12 months of LT4 treatment. In addition, we studied the consequences of the mutation for TRα1/2 receptor function *in vitro*.

Results

At 18 months of age, the patient presented with axial hypotonia, delayed motor development, severe growth retardation and abnormally elevated T3/T4 ratios. RTHα was suspected and concomitantly a c.632A>G/p.D211G missense mutation was identified, affecting both the TRα1 and TRα2 proteins. This mutation was also found in the girl's father. LT4 treatment was started, resulting in a marked improvement of her hypotonia, motor skills and growth. Functionally, the missense mutation led to a decreased transcriptional activity of TRα1, which could be overcome by higher T3 levels *in vitro*. The mutant TRα1 showed a moderate dominant negative activity on WT TRα1. In contrast, WT TRα2 and mutant TRα2 had negligible transcriptional activity and showed no dominant-negative effect over TRα1.

Conclusions

This report describes the phenotype of a young RTHα patient with a mild TRα mutation before and during early LT4 treatment. Treatment had beneficial effects on her muscle tone, motor development and growth.

Introduction

Thyroid hormone (TH) is essential for normal development and metabolism. The majority of TH actions is mediated by binding of triiodothyronine (T3), the bioactive form of TH, to nuclear thyroid hormone receptors (TRs). TRs bind as heterodimers with Retinoid X Receptors (RXRs) to TH response elements (TREs), thereby regulating target gene expression.[1,105,108] TRs are encoded by 2 genes (*THRA* and *THRB*), from which different isoforms are generated, with TRα1, TRβ1 and TRβ2 being highly homologous T3 binding isoforms. TRα1 is preferentially expressed in the CNS, bone, heart and intestine while the non-T3 binding isoform TRα2 is more widely expressed. TRβ1 is considered the major isoform in liver, kidney, and thyroid. TRβ2 has a more restricted expression pattern regulating neurosensory development as well as the hypothalamus-pituitary-thyroid (HPT) axis.[67,108,109]

For over 20 years it has been known that heterozygous mutations within 3 hotspots of the ligand-binding domain (LBD) of TRβ result in resistance to thyroid hormone β (RTHβ), a clinical syndrome characterized by elevated serum TH levels, a non-suppressed TSH, and a variable phenotype comprising goiter, tachycardia and increased energy expenditure. Recently, the first patients with RTHα due to mutations in the LBD of TRα1 have been identified. These patients are characterized by growth retardation, variable motor and cognitive defects, macrocephaly and abnormal thyroid function tests (elevated T3/T4 and T3/rT3 ratios with normal TSH levels).[151,153,178,188–191] Recently, *Moran et al*. identified the first family with a mutation in both TRα1 and TRα2.[192] Patients in this family displayed a similar clinical and biochemical phenotype as patients with a mutation in TRα1 alone, suggesting that the mutation in TRα2 does not contribute to the nature or severity of the phenotype.[192,193] Although only a few patients with RTHα have been identified so far, the severity of the phenotype appears to be related to the location and type of the mutation in TRα1. Considering the crucial role of TH during development, RTHα patients may benefit differently from levothyroxine (LT4) treatment depending on the severity of the mutation and the timing of treatment.[151,153,178,188,190–192]

In this study, we describe the youngest patient identified with RTHα so far. She was diagnosed with a mild mutation in both TRα1 and TRα2 and was treated with LT4 from the age of 18 months onwards. Beneficial effects of treatment on different parameters such as motor development, growth and thyroid function tests are supported by *in vitro* data showing that the reduced sensitivity of the mutant TRα1 towards T3 could be overcome by higher T3 concentrations.

Patients, materials and methods

Index patient

Because of suspected central hypothyroidism, the index patient was referred to a pediatric endocrinologist, who conducted extensive thyroid function tests showing low FT4 and rT3 levels, with a high T3 and a normal TSH concentration. Based on the clinical phenotype and this typical biochemical profile, a mutation in THRA was suspected.

Clinical and biochemical assessment

Written informed consent was obtained from the parents of the index patient. The study was approved by the Medical Ethics Committee of the Amsterdam Medical Center.
From the age of 18 months onwards, the girl underwent detailed physical examinations (growth parameters, head circumference) by the pediatric endocrinologist once every 2–3 months. In addition, biochemical measurements (T4, FT4, T3, rT3, TSH, thyroglobulin, TBG, IGF-1, IGFBP-3, SHBG, ferritin, complete blood count), imaging (skeletal X-ray) and motor developmental tests (Alberta Infant Motor Scale (AIMS)[194] and the Bayley Scales of Infant and Toddler Development (Bayley III-NL))[195] were performed at specific time points.

Genetic analysis

Sequencing of exons 6–9 of *THRA* [GenBank: NM_199334.3] was performed as previously described.[178] The sequence results were compared to the reference sequence of *THRA*. To exclude PCR errors, the amplification and sequencing of the exon carrying the mutation was repeated.

Functional characterization of the mutant TRα1 and TRα2

DNA constructs and mutagenesis

The mammalian expression vector pcDNA3-FLAG-TRα1 was used, containing the full length coding sequence of human TRα1 with a 5'-FLAG tag and optimized Kozak sequence as previously described.[151] The pcDNA3-FLAG-TRα2 expression vector was generated according to the same methods (see Supplemental Table 1 for primers). The RXRα plasmid was generated by cloning the full length coding sequence of human RXRα into the *EcoRI* and *XbaI* sites of the pcDNA3 expression vector (see Supplemental Table 1 for primers).
Mutations in TRα1 and TRα2 were generated by site directed mutagenesis using the QuickChange II Mutagenesis kit (Agilent Technologies, Amstelveen, NL) according to the manufacturer's instructions. The presence of the introduced mutations was confirmed by sequencing.

To study the transcriptional activity of WT and mutant TRα1 and TRα2, a construct was used containing a DR+4 TRE-dependent firefly luciferase reporter and a control renilla luciferase reporter (pdV-L1; gift from Dr. W.S. Simonides, VUMC, NL[129]).

Cell culture and transfection

JEG3 cells were maintained in Dulbecco's modified Eagle's/F12 medium supplemented with 9% FBS and 100 nM Na_2SeO_3 at 37˚C and 5% CO_2. To deplete cells of TH, charcoal-treated FBS (CT-medium) was used instead during 48 hours. Cells were transfected at 80–90% confluence with 100 ng pdV-L1 luciferase-renilla reporter construct together with 100 ng WT or mutant TRα1, or the combination of 50 ng WT and 50 ng mutant TRα1 using Xtreme Gene 9 transfection reagent (Roche Diagnostics, Almere, NL) according to the manufacturer's protocol. To explore the possible effect of the mutation on TRα2 function, WT TRα1 was co-transfected with WT or mutant TRα2 in different ratios (1:1, 1:10, 1:50).

Luciferase assays

Transcriptional activity of WT and mutant receptors was determined using the Dual-Glo Luciferase kit (Promega, Leiden, NL), as described before.[153] The luciferase activity was normalized to the renilla activity to adjust for transfection efficiency. The results are presented as the means ±SEM of at least three or more independent experiments, carried out in triplicate.

Immunoblotting

To determine expression of WT and mutant TRα1 and TRα2, transfected cells were lysed in ice-cold RIPA buffer supplemented with the Complete Protease Inhibitor cocktail (Roche Diagnostics, Almere, NL). The lysates were cleared by centrifugation for 5 min at 3000 rpm and the protein concentration of the supernatant determined by bicinchoninic assay (BCA) (Fisher Scientific, Landsmeer, NL). Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in TBST / 5% milk and probed overnight at 4˚C with a 1:1000 dilution of the FLAG M2-antibody (F1804 Sigma-Aldrich). Bound antibody was detected with a HRP-conjugated goat-anti mouse antibody ((#172–1011 Bio-Rad Life Science, Veenendaal, NL) and visualized by enhanced chemiluminescence using the Alliance 4.0 Uvitec platform (Uvitec Limited, Cambridge, UK).

Electrophoretic Mobility Shift Assays (EMSA)

Direct binding of WT and mutant TRα receptors to DNA was tested by EMSA, using a double stranded overlapping oligonucleotide probe labelled with a fill-in reaction using Klenow fragment in the presence of ^{32}P-labeled dCTP.[196] The sequences of the oligonucleotides

used for the synthesis of the DR+4 TRE-containing probe are listed in the Supplemental Table 1. The ^{32}P-labeled probe was purified using NucAway Spin Colums (Life Technologies, Bleiswijk, NL). Receptor proteins were transcribed and translated *in vitro* using the TnT Quick Coupled Transcription/Translation System (Promega, Leiden, NL), according to the manufacturer's protocol. The binding assays were performed according to previously described methods.[197,198] Radioactive spots were visualized by autoradiography.

Statistical tests

Statistical differences between WT and mutant receptors were calculated using Student's t-test. *P*-values < 0.05 were considered statistically significant.

Results

Case report and clinical assessment of the index patient at baseline

The girl was born at term by vacuum extraction after an uncomplicated pregnancy. Birth weight was 4000 g, and Apgar scores 5, 7 and 9 after 1, 5 and 10 minutes, respectively. After an uneventful neonatal period, including a normal neonatal congenital hypothyroidism screening result (T4 62 nmol/L [-1,3 SD], TSH 1 mIU/L), the girl displayed hypotonia and slow motor development, whereupon she was referred to a pediatric physical therapist. At first consultation at the age of 8.5 months the hypotonia was found to be predominantly "axial". Motor developmental testing,[194] yielded an AIMS score below the 5th percentile, indicating severe delay in motor development.

At the age of 10.5 months, the girl was referred to a pediatric neurologist for lack of progression in motor development and persisting hypotonia. In the absence of "alarm" signs suggestive of serious neuromuscular disease, and because cognitive development was judged normal, diagnostic testing was postponed and frequent physical therapy (PT) was continued. At the age of 12 months, the girl rolled over from tummy to back and vice versa for the first time, a milestone normally occurring at 9 months[199]), but she was still unable to crawl or pull herself up to a standing position, characteristic milestones for 12 months[199]). Although the AIMS was not routinely performed after the first PT consultation the physical therapist reported no clear improvement up to the age of 18 months (based on home video fragments filmed between the ages of 17 and 18 months the physical therapist estimated the AIMS score at that age between 20 and 30, still far below the 5th percentile; Figure 2D).

Because of persistent hypotonia, lack of improvement in motor development and mild speech delay (babbling and the use of only one word), additional investigations were performed around the age of 17 months. A full blood count and blood chemistry (including liver enzymes and creatine kinase), and electromyography were normal.

However, repeated laboratory testing revealed FT4 concentrations (9.0–9.8 pmol/L) just below the lower limit of the reference interval (10.0–20.0 pmol/L) in the presence of normal TSH concentrations (2.0–5.6 mIU/L; reference interval 0.48–4.6 mU/L). At this point, she was referred to a pediatric endocrinologist, at the age of 18 months for suspicion of central hypothyroidism. The medical history revealed mild constipation from the age of 6 months onwards, but no other problems. The family history revealed primary hypothyroidism in the maternal grandmother, but no thyroid or pituitary disorders in other family members or in the father's family.

On physical examination height was 73 cm (-2.77 SD), weight 10.6 kg (+2.11 SD), BMI 19.89 (+2.17 SD) and head circumference 47 cm (0 SD) (Figures 2A-2B). The face and nasal bridge were broad, but there was no macroglossia. Skin tags were absent. Neurological examination showed impaired trunk balance with axial hypotonia and more pronounced hypotonia in the arms than in the legs. Repeated, but more extensive thyroid function testing showed low FT4, T4 and rT3, high T3 and normal TSH concentrations, and increased T3/T4 and T3/rT3 ratios (Table 1), supporting clinical suspicion of a mutation in THRA.

Genetic assessment

Sequencing of *THRA* indicated that the index patient and her father were heterozygous for a nucleotide substitution (c.632A>G), resulting in an aspartic acid to glycine substitution at codon 211 (p.D211G) in both TRα1 and TRα2 (Figure 1). This mutation is not present in public databases.

Clinical assessment of the index patient during LT4 treatment

Because of the presumptive diagnosis of central hypothyroidism, awaiting the results of *THRA* mutation analysis, treatment with LT4 was started with a daily dose of 3.5 µg/kg body weight. The dose was gradually increased to a final dose of 62.5 µg (5.25 µg/kg body weight). After 6 months of LT4 treatment (chronological age of 24 months), the index patient showed clear improvement in motor development: her hypotonia had almost disappeared and she was able to crawl, sit and walk independently, albeit with a broad gait. Repeat assessment by the referring pediatric neurologist showed no clear abnormalities. AIMS testing by the same physical therapist yielded a score of 54, corresponding to the 50th percentile at 12 months of age (Figure 2C). In addition, the parents found their daughter to be livelier, less tired, and her bowel movements had normalized to once daily. Her speech was still slightly delayed (use of a few words, but not yet two-word phrases (milestone: 24 months[199])). Length had increased to -1.98 SD and head circumference +0.76 SD (Figure 2A,B). Bone age was delayed (9 months at chronologic age of 21 months). On LT4 treatment, plasma FT4 normalized and TSH was suppressed (Table 1).

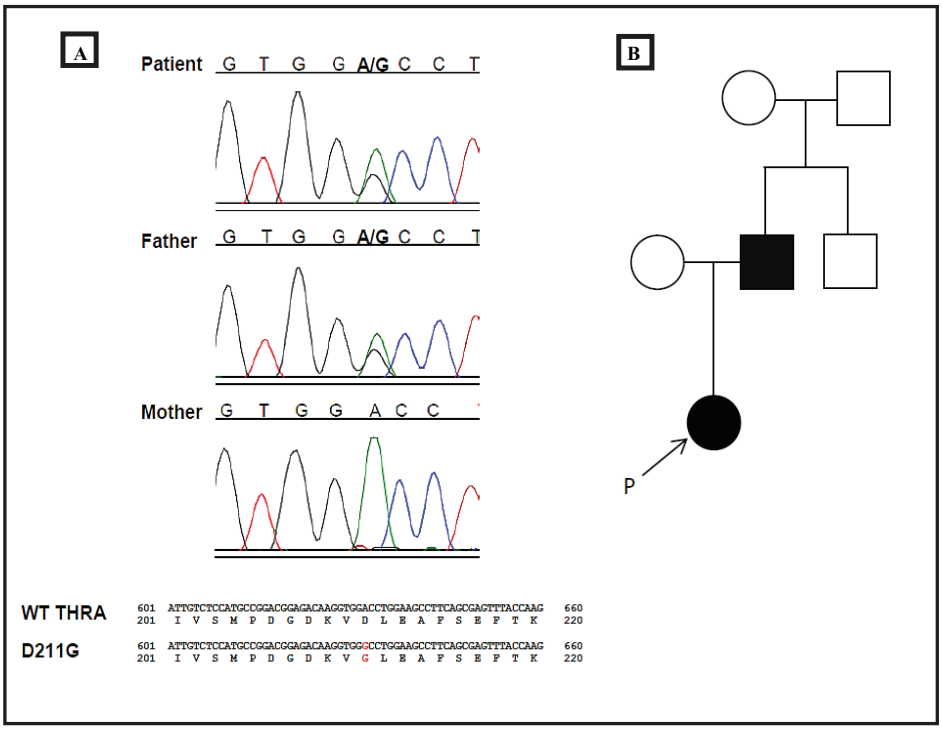

Figure 1. Molecular analysis of THRA in the index patient and her father.
(A) Sequence analysis of exon 7 of THRA shows the nucleotide substitution (c.632A>G), corresponding to an aspartic acid to glycine substitution at codon 211 (D211G) in both the index patient and her father, which is absent in her mother **(B)**.

Table 1. Biochemical characteristics of the index patient before and on levothyroxine treatment.

Biochemical measurements in the index patient				
Variable	Reference values	Before treatment	On treatment (6 months)	On treatment (12 months)
T4	70 – 150 nmol/L	110	145	165
FT4	10 – 23 pmol/L	9.0	15.5	19.1
T3	1.3 – 2.7 nmol/L	3.6	3.9	5.0
rT3	0.11 – 0.44 nmol/L	0.09	0.18	0.19
TSH	0.5 – 5.0 mE/L	4.40	< 0.01	0.01
T3/T4 (x100)	1.42 – 3.05	3.27	2.69	3.03
T3/rT3	3.1 – 13.0	40	21.67	26.32
Thyreglobulin	0.0 – 45 pmol/L	19.0	5.0	6.0
TBG	200 – 650 nmol/L	800	400	420
IGF-1	3.0 – 20 nmol/L	1.0	8.0	8.0
Hemoglobin	6.0 – 9.0 mmol/L	6.2	6.7	
MCV	70 – 85 fL		85.1	
Ferritin	15 – 150 µg/L		312	

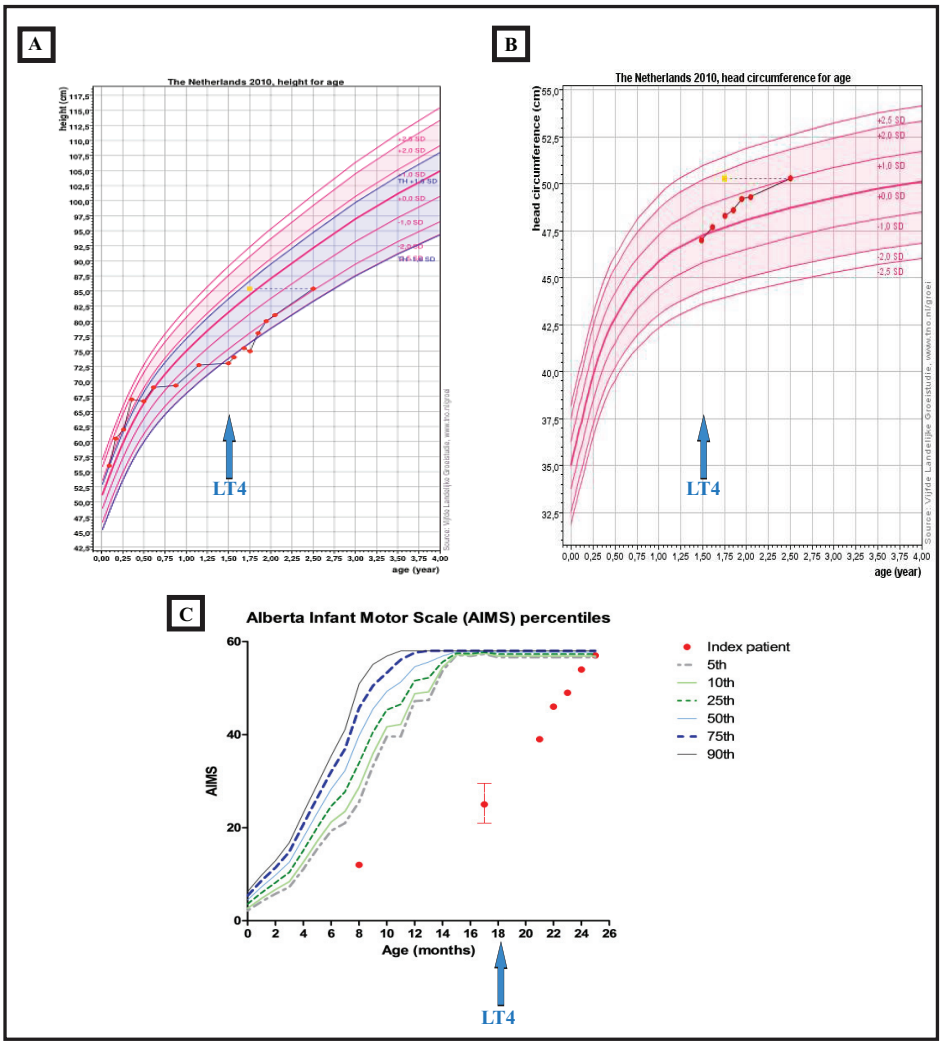

Figure 2. Phenotypic characteristics of the index patient.
(A) The index patient's growth chart showing deflecting growth between the ages of 6 and 18 months, but clear catch-up growth after the start of levothyroxine treatment at the age of 18 months. **(B)** Chart showing the index patient's head circumference between the ages of 18 and 30 months. **(C)** The index patient had a severe delay in motor development, as measured by the Alberta Infant Motor Scale (AIMS), which improved after the start of treatment.

After one year of LT4 treatment, still at a dose of 62.5 µg per day, the now 30 months old girl showed further progress in motor development, with Bayley III-NL scores[195] for gross and fine motor skills of 50 and 41, corresponding to developmental ages of 19 and 25 months, respectively. Speech development had also improved: the girl was now able to say two-word phrases (milestone: 24 months[199]). Formal neurological examination at the age of 32 months revealed only very mild hypotonia, which disabled her to

change from lying to sitting position. In order to compensate, she rolled over to her tummy and pushed herself to a sitting position. Further assessment showed normal gross and fine motor skills and symmetric reflexes. In addition, length had increased to -1.92 SD, head circumference to +1.84 SD, and bone age to 21 months (chronological age 30 months), respectively.

Clinical assessment of the girl's father

The father of the patient has the same mutation and has not been treated to date. No detailed data on childhood development are available, but he reported delayed puberty. At the age of 15 years he had four epileptic episodes for which he was treated with valproic acid. Furthermore, he suffered from constipation (hard stools 2 – 3 times a week). Physical examination showed coarse facies, macrocephaly (+ 2.5 SD), a short stature (-1.19 SD) and, an increased body mass index (28.3 kg/m^2). Blood pressure (135/85 mmHg) and bone mineral density (femoral head: Z-score -0.3, T-score -0.5; lumbar spine: Z-score 0.9, T-score 0.9) were normal. In addition, neuropsychological evaluation showed normal performances on memory, language, attention and executive function tests together with a normal processing speed. Lastly, his biochemical profile (abnormal T3/T4 and T3/rT3 ratios with a normal TSH and a mild anemia; Table 2) fitted well with the previous patients identified with RTHα.[151,153,178,188,189,192]

Table 2. Biochemical characteristics of the girl's father.

Biochemical measurements in the girl's father		
Variable	Reference values	
T4	70 – 150 nmol/L	85
FT4	10 – 23 pmol/L	10.1
T3	1.3 – 2.7 nmol/L	2.25
rT3	0.11 – 0.44 nmol/L	0.12
TSH	0.5 – 5.0 mE/L	1.60
T3/T4 (x100)	1.42 – 3.05	2.65
T3/rT3	3.1 – 13.0	18.75
Thyroglobulin	0 – 45 pmol/L	13
TBG	200 – 650 nmol/L	
IGF-1	8 – 41 nmol/L	24
Hemoglobin	8.5 – 10.5 mmol/L	7.3
MCV	80 – 100 fL	98.2
Ferritin	25 – 300 µg/L	272

Functional analysis of mutant TRα1 and TRα2

To determine the effect of the mutation on TRα1 function, we expressed FLAG-tagged WT or D211G-TRα1 in JEG3 cells and determined T3-dependent transcriptional activity.

In luciferase assays, the D211G mutant TRα1 showed a reduced apparent affinity for T3 compared to WT TRα1 (EC_{50} 10.1 nM T3 for D211G TRα1 vs. 0.49 nM T3 for WT TRα1,

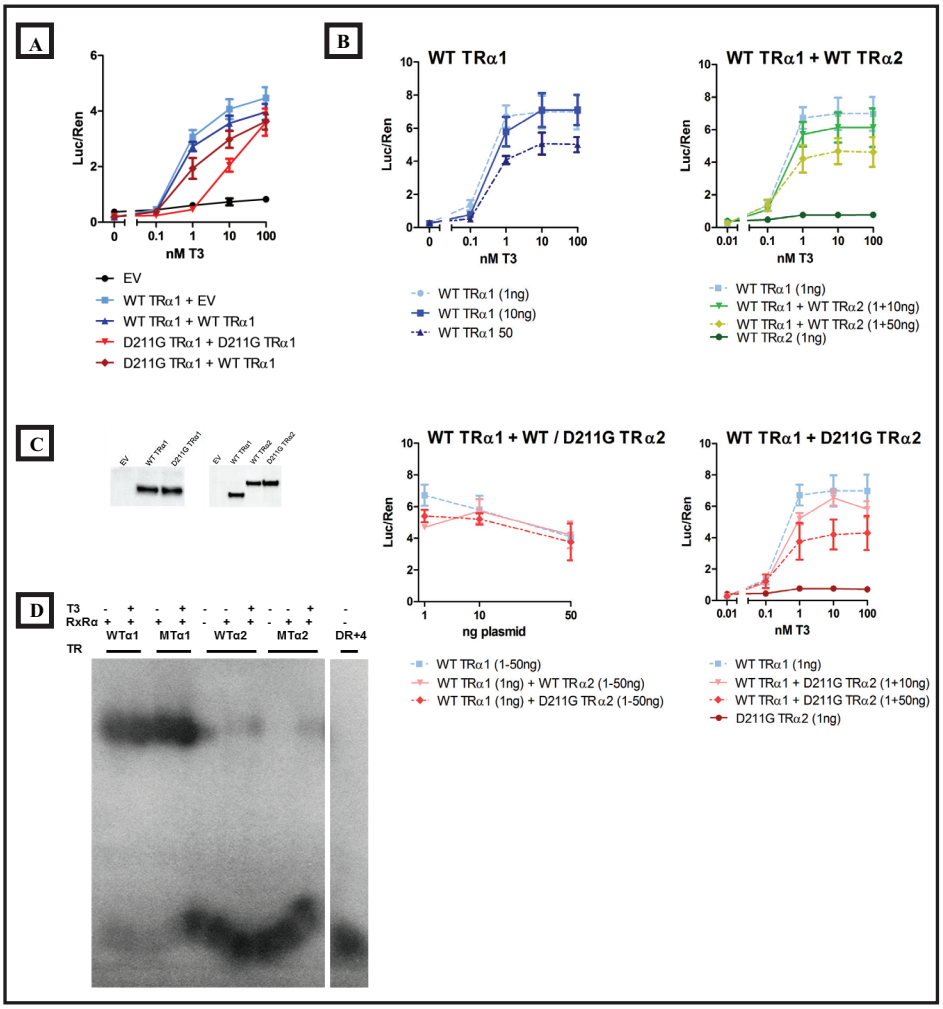

Figure 3. Functional analysis of D211G mutant TRα1 and TRα2.
(A) The transcriptional activity of WT and D211G TRα1 was tested in JEG3 cells transfected with a T3-dependent promoter-reporter construct. Mutant TRα1 had a reduced affinity for T3 compared to WT TRα1 at low T3 concentrations, while at higher T3 concentrations the maximal transcriptional activity was similar. When co-expressed, mutant TRα1 showed no dominant-negative effect on WT TRα1. Results are the means ± SEM of 3–5 experiments. **(B)** The biological activity of TRα2 and the possible interaction with TRα1 was studied by transfecting cells with an increasing amount of WT TRα1 (1–50ng) and increasing ratios (1:1 – 1:50) of WT or D211G TRα2. WT TRα2 and mutant TRα2 showed no transcriptional activity and both TRα2 receptors hardly had any dominant-negative effect over TRα1. Results are the means ± SEM of 3–8 experiments. **(C)** Expression of the TR proteins was verified by western blot analysis, showing a similar expression of WT and mutant TRα1 and TRα2. **(D)** Electrophoretic mobility shift assay showing the binding of hormone bound or unbound WT and D211G TRα1 to a direct repeat (DR+4) thyroid response element.

$p < 0.001$). However, the D211G mutant had a similar maximal transcriptional activity as WT TRα1 (Figure 3A). When co-expressed, the D211G mutant TRα1 had a moderate dominant-negative effect on WT TRα1 function (Figure 3A).
Independent of the T3 concentration, WT TRα2 and D211G TRα2 showed no transcriptional activity. Co-transfection of WT TRα1 with increasing amounts of WT or mutant TRα2 (1:1, 1:10, 1:50) suppressed transcriptional activity, but the same was the case after increasing the amount of WT TRα1 plasmid alone (Figure 3B). This indicates that both WT and mutant TRα2 exert little or no dominant-negative effect over TRα1.
A similar expression of WT TRα1, WT TRα2 and D211G TRα2 proteins was confirmed by immunoblotting lysates for FLAG (Figure 3C).
In addition, we tested the binding capacity of WT and mutant TRα1 and TRα2 to a ^{32}P-labeled DR+4 TRE probe by EMSA in the absence or presence of RXRα and T3. WT and mutant TRα1 bound similarly to the TRE as heterodimers with RXRα independent of T3, whereas no binding of WT TRα2 or mutant TRα2 to the probe was detected under any of the conditions tested (Figure 3D). In the absence of RXRα both WT and mutant TRα1 still bound to the TRE, but the binding was less pronounced (data not shown).
Overall, these results suggest that D211G TRα1 has a reduced affinity for T3, while DNA binding and expression levels are similar to WT TRα1. At high concentrations of T3, maximal transcriptional activity of the mutant is the same as for WT TRα1. In contrast to TRα1, both WT TRα2 and D211G TRα2 show no transcriptional activity nor binding to the DR+4 TRE.

Discussion

In the current study we describe the effects of early LT4 treatment in a very young girl with a mild missense mutation in TRα1/2. We report beneficial effects during treatment on her muscle tone, motor development and growth. This is in line with our *in vitro* data showing that the reduced transcriptional activity of the mutant receptor could be overcome by higher T3 concentrations, and that D211G TRα1 has a moderate dominant negative effect on WT TRα1. The D211G mutation in TRα2 does not influence the function of WT TRα1 or TRα2 and does not seem to contribute to the phenotype of the patient.
Although the index patient had some clinical characteristics associated with hypothyroidism (growth retardation, and a broad face and nasal bridge), her hypotonia and delay in motor development were the prime reasons for referral and consultation. Because of presumed central hypothyroidism, based on normal TSH with low-normal FT4 levels, LT4 treatment was started at the age of 18 months. LT4 treatment had a beneficial effect on the girl's development: her muscle tone improved and her motor

development accelerated. In addition, during treatment she showed catch up growth and a normalization of her FT4 and rT3 levels. The suppressed TSH concentrations upon LT4 treatment are in line with previous reports[153] and indicate an intact HPT-axis feedback, which is predominantly regulated via TRβ2.[200] Our *in vitro* data support the developmental benefit of LT4 treatment, since transcriptional activity of the mutant TRα1 could be restored *in vitro* by higher T3 concentrations.

The fact that the girl's father reached adulthood without major complications associated with the TRα1 D211G mutation supports the relatively mild character of this mutation with a decreased but not absent affinity for T3. The difference in severity between the developmental phenotype of the father and the daughter is remarkable, but similar to RTHβ in which the severity of the phenotype also varies among different subjects carrying the same mutation.[7] The reasons for this variability are not yet fully understood, but may result from genetic variability in other genes, such as cofactors involved in TH action.

Anemia is a frequent characteristic in RTHα and was only present in the father of the index patient. Usually, it concerns a normocytic normochromic anemia however, in three cases the MCV was raised.[151,188,193] Several studies in humans have indicated an association between hypothyroidism and anemia.[201,202] In addition, data from animal models point towards an important role for TRα in erythropoiesis.[203–206] The molecular mechanisms underlying the anemia in RTHα patients remain to be elucidated.

The phenotype of the girl shows similarities with that of heterozygous TRα1-R384C mutant mice, having a mutant receptor with a 10-fold reduced affinity for T3.[207] Affected animals show delayed postnatal development together with locomotor dysfunctions. Interestingly, treatment with supra-physiological TH doses in the postnatal period, but not in adulthood, substantially improved motor function.[208] These TRα1-R384C mutant mice also suffer from growth retardation, which is overcome in adulthood since they reach a near-normal size.[111,207]

The phenotype of the index patient and her father resemble the clinical characteristics of previously described RTHα patients with a mutation in TRα1 alone.[151,153,178,188–190] The D211G mutation in TRα2 does not seem to contribute to this phenotype, which is in line with what has been described in three patients with the A263V mutation, also affecting TRα1 and TRα2.[191,192] Our findings are also in line with the phenotype of the TRα2KO mice, since the phenotypic changes in these mice are most likely not attributable to the loss of TRα2, but to a compensatory overexpression of TRα1 in that particular mouse model.[209] In contrast, a recently described patient[193] with a N359Y mutation in TRα1/2 showed a distinct clinical phenotype consisting of severe bone malformation, chronic diarrhea, macrocytic anemia and hypercalcemia. To date it remains unclear whether this phenotype is the result of the mutation in *THRA* alone. Our *in vitro* results support a lack of effect of the mutation in TRα2, since WT and D211G TRα2 showed no transcriptional

activity both in the absence or presence of T3. Co-transfection of WT TRα1 with large amounts of WT or D211G mutant TRα2 suppressed the function of WT TRα1 in the same way as when we increased the concentration of WT TRα1 plasmid alone. This suggests that the suppressive effect is caused by an increased amount of transfected plasmid and not by a dominant-negative effect of the TRα2 receptors towards WT TRα1.

The lack of effect of WT or mutant TRα2 on the transcriptional activity of TRα1 is explained by the observation that TRα2 does not bind to the TRE (DR+4) we used in our luciferase assays, independent of the absence or presence of RXRα. Under the conditions of our experiments, competition for binding to the TRE is therefore absent and so is a suppressive effect of TRα2 on TRα1 function. These findings are supported by the study of *Liu et al.*, describing that the possible dominant-negative effect of TRα2 on TRα1 is not attributable to competitive binding to TREs or inadequate formation of heterodimers with RXRα.[210]

The fact that the girl's father has RTHα with a relatively mild phenotype, which was detected only because of severe developmental delay of his daughter, supports the hypothesis that the incidence of RTHα could be much higher than currently estimated. Suggestively, the RTHα incidence could correspond to the incidence of RTHβ (1:40,000),[7] given the strong homology between the TRα1 and TRβ1 receptors. Moreover, although so far only a few mutations in TRα have been reported, they seem to cluster in a few hotspots similar to the pattern of TRβ mutations.[211] Lastly, the paternal inheritance of the TRα mutation in this family, and maternal and paternal inheritance of TRα mutations in three other families,[153,178,189,192] suggests that transmission of TRα mutations from parents to offspring is less impaired in humans than in mice.[112]

This case study illustrates that RTHα patients with mild mutations, especially when diagnosed at a young age, are likely to benefit from LT4 treatment. This is supported by a previous case[192] as well as by studies in TRα mutant mice.[207,212]

Since the index patient was initially suspected of having central hypothyroidism, we advocate a low threshold to measure T3 (and rT3) in the work-up of children with unexplained growth retardation or developmental delay who have a low or low-normal FT4 in combination with a normal TSH.

Acknowledgements

We thank Leontien van der Aa and Erna Langius for their contribution to the clinical assessment of the index patient.
A.L.M. van Gucht, M.E. Meima and R.P. Peeters are supported by a Zon-MWTOP Grant (number 91212044) and an Erasmus MC MRACE Grant.

Author Disclosure Statement

The authors have nothing to disclose.

Supplementary Table 1. Primers used for generation of pcDNA3-FLAG-TRα2 expression vector, RXRα plasmid and DR+ TRE-containing probe.

	Forward primer (5′-3′)	Reverse primer (5′-3′)
pcDNA3-FLAG-TRα2	GTAGAATTCTGGCCGCAGAAATGGACTACAAAGACGATGACGACAAGATGGAACAGAAGCCAAGCAAGG	GTACTCGAGACTCACTTTCAGGGAGAGGCTGCATTGCC
RXRα	GTAGAATTCTGGCCGCAGAAATGGACACCAAACATTTCCTGCC	CTATCTAGACTAAGTCATTTGGTGCGGCGC
DR+4 TRE (half sites underlined)	ATCCCCGGGCGAGCTCTTCAGGTCACTTCAGGTCACCGGG	GAAGAGCTCGCCCGGTGACCTGAAGTGACCTGAAGAGCTC

DIVERSE GENOTYPES AND PHENOTYPES OF THREE NOVEL THYROID HORMONE RECEPTOR ALPHA MUTATIONS

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Journal of Clinical Endocrinology and Metabolism 2016

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Abstract

Context

Recently, several patients with RTHα due to T3 receptor alpha (TRα) mutations were identified. The phenotype of these patients consists of varying degrees of growth impairment, delayed bone, mental and motor development, constipation, macrocephaly, and near-normal thyroid function tests.

Objective

To describe the clinical phenotype of three new families with RTHα and thereby gain more detailed knowledge on this novel syndrome.

Design, setting, participants

RTHα was suspected in 3 index patients from different families. Detailed clinical and biochemical assessment, imaging and genetic analyses were performed in the patients and their relatives. In addition, functional consequences of TRα mutations were investigated *in vitro*.

Results

We studied 22 individuals from 3 families, and identified 10 patients with heterozygous TRα mutations: C380fs387X, R384H, and A263S, respectively. The frame-shift mutation completely inactivated TRα, whereas the missense mutations produced milder defects. These mutations were associated with decreasing severity of the clinical phenotype: the patient in family 1 showed severe defects in growth, mental and motor development, whereas the 7 patients in family 3 only had mild clinical features. The most frequent abnormalities were anemia, constipation, and a delay in at least one of the developmental milestones. Serum (F)T3 ranged from high-normal to high, and serum (F)T4 and rT3 from normal to low. TSH levels were normal in all patients.

Conclusions

This large case series underlines the variation in the clinical phenotype of RTHα patients. RTHα should be suspected in subjects when even mild clinical and laboratory features of hypothyroidism are present along with high/high-normal (F)T3, low/normal (F)T4, and normal TSH.

Introduction

Genetic disorders associated with impaired sensitivity to thyroid hormone (TH) include 1) resistance to TH (RTHβ) due to mutations in *THRB* which codes for the T3 receptor TRβ1 and TRβ2 isoforms, 2) the Allan-Herndon-Dudley syndrome (AHDS), a severe neurological disorder caused by mutations in the TH transporter MCT8, and 3) a multisystem disorder caused by mutations in SBP2, a protein essential for the production of selenoproteins, including the TH activating and inactivating deiodinases.[7,106,107,213] Until 2012, no mutation was reported in *THRA* encoding T3 receptor subtypes TRα1 and TRα2. Products of the *THRB* and *THRA* genes show differential tissue expression.[214–218] TRβ1 is the predominant T3 receptor in liver, kidney, and thyroid, and TRβ2 is exclusively expressed in hypothalamus, pituitary, retina and cochlea. On the other hand, growth, development, and function of brain, bone, heart, and intestine are mainly dependent on interaction of T3 with TRα1. TRα2 does not bind T3 and its function is enigmatic.

Since 2012, 15 patients from 10 families have been reported with the RTHα syndrome due to mutations in *THRA*.[130,151,153,188,193,219–221] The clinical pictures and TH profiles associated with RTHα and RTHβ are consistent with the expression patterns of *THRA* and *THRB*. Elevated serum T3 and T4 with non-suppressed TSH levels are typical for RTHβ. Symptoms are usually mild but may include goiter, tachycardia, atrial fibrillation, mild mental retardation, hyperactivity, and increased resting metabolic rate.[7,106,107] Patients with RTHα have varying degrees of developmental retardation, growth impairment, and constipation, with low to low-normal (F)T4, normal to high (F)T3, and normal to mildly elevated TSH levels.[107,130,151,153,188,191,219–221]

Here, we present the clinical and laboratory characteristics of the largest case series so far with 3 novel *THRA* mutations in a total of 10 patients resulting in a wide spectrum of RTHα phenotypes. More awareness of the variation in the clinical presentation of RTHα is likely to result in the identification of additional RTHα patients.

Patients and Methods

Patients

Three index patients with symptoms and signs suggestive of hypothyroidism associated with near-normal TH levels and their families were included. Detailed information regarding developmental milestones and symptoms of hypothyroidism were collected. The subjects underwent physical examination, including anthropometric measurements, biochemical tests, imaging, genetic studies, and neurodevelopmental tests. The study was approved by the Medical Ethics Committee of Dokuz Eylül University. Written informed consent was obtained from all subjects and/or their parents.

Neurodevelopmental and neuropsychological evaluation

Developmental state was evaluated with the Bayley Scales of Infant and Toddler Development, Second Edition (Bayley II) in children < 3 years.[222] Older children were tested with Wechsler Intelligence Scale for Children-Revised (WISC-R) intelligence tests.[223] Adult patients were assessed with Wechsler Adult Intelligence Scale to determine their IQ score and classification was done based on Wechsler classification system.[224] A neuropsychological test battery composed of Mini-Mental State Examination for general cognitive functioning, Oktem Auditory-Verbal Learning Test, Rey-Osterreith Complex Figure Tests for memory and visuospatial skills, and Stroop and Trail Making Tests for executive functioning were applied.[225–227] Each test has been shown to be reliable and valid in the Turkish population and the details of the procedures can be found elsewhere.[228–230] In addition, adult patients were examined for lifetime psychiatric diagnoses and family history.

Laboratory and imaging studies

The thyroid status of all subjects was initially evaluated using measurements of FT4, FT3 and TSH in İzmir (Roche Diagnostics, Mannheim, Germany). Total cholesterol, creatinine kinase (CK), complete blood count, ferritin, SHBG, IGF-1, and IGFBP-3 were measured from blood samples obtained between 8 AM and 10 AM after an overnight fast using routine laboratory methods. Available serum samples were also assayed for TSH, FT4, T4, T3 (Vitros ECI technology, Ortho-Clinical Diagnostics, Beerse, Belgium), and rT3 (radioimmunoassay, Zentech, Angleur, Belgium) levels in Rotterdam. Hip and skull X-rays were obtained.

Identification and functional analysis of THRA mutants

THRA was analyzed for mutations as described.[221] FLAG-tagged WT and mutant TRα1 and TRα2 cDNA clones in pcDNA3 were obtained as described.[221] Transcriptional activity of WT and mutant TRα1 was assessed using a reporter construct expressing firefly luciferase (LUC) under control of a T3 responsive promoter and renilla luciferase (REN) under control of a constitutive promoter as described.[221] Expression of WT and mutant TRα1 and TRα2 was determined by immunoblotting using anti-FLAG antibody as described.[221]

Statistical tests

Statistical differences between WT and mutant receptors were calculated using a Student's t-test. *P*-values < 0.05 were considered statistically significant.

Results

Clinical Assessment

Among 3 families, 10 patients were identified with *THRA* mutations: 6 children and 4 adults, 4 males and 6 females, age range 8 months – 56 years. The pedigrees, photographs and mutations of the patients are shown in Figure 1. Table 1 presents an overview of the clinical data, and Table 2[231] summarizes the laboratory data. Results of neurological and neuropsychological tests are provided in Supplemental Table 2. Skull X-ray photographs are presented in Supplemental Figure 2. The families are presented in chronological order of diagnosis and evaluation of cases.

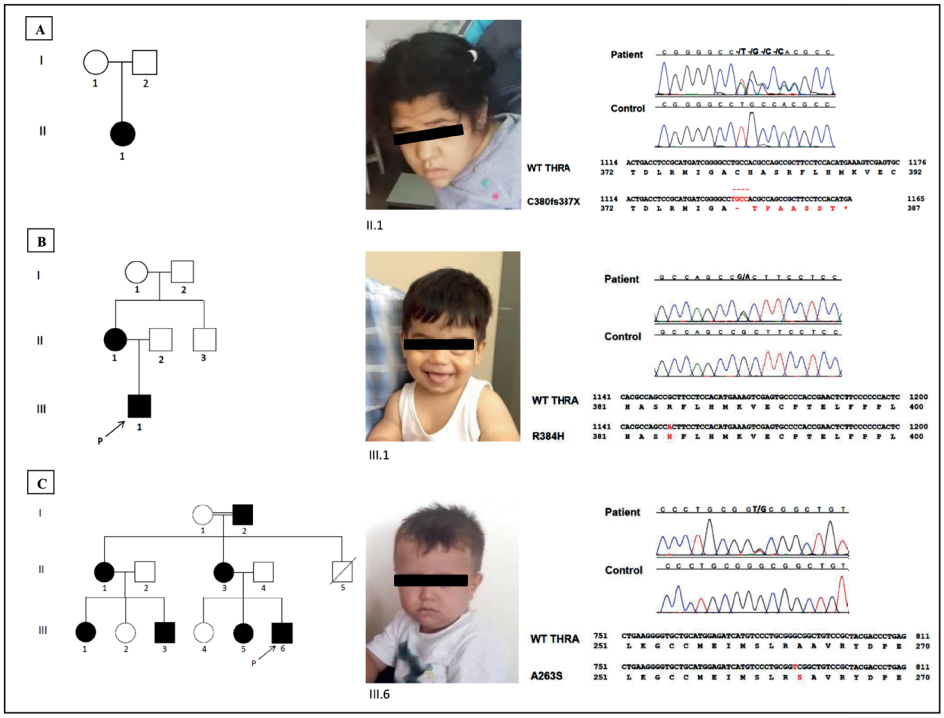


Figure 1. Pedigree and molecular analysis of THRA in the three index patients.
(**A**) Sequence analysis of exon 9 of *THRA* shows a deletion of 4 nucleotides (c.1138–1141delTGCC), resulting in a deletion and frameshift at codon 380 and an early stop at codon 387 (p.C380fs387X). This mutation is only present in the index patient (1.II.1). **(B)** In both the index patient (2.III.1) and his mother (2.II.1) a nucleotide substitution (c.1151G>A) lead to an arginine to histidine substitution at codon 384 in TRα1 (p.R384H). **(C)** The index patient (3.III.6) and 6 other members of this family (3.III.1, 3.III.3, 3.III.5, 3.II.1, 3.II.3, 3.I.2) were heterozygous for a nucleotide substitution in *THRA* (c.G787T), leading to an alanine to serine substitution at codon 263 (p.A263S).

Table 1. Clinical characteristics of 3 families with THRA mutations

	Age Yrs	M/F	Signs of hypothyroidism	Height SDS	BMI or WFH SDS	HCF SDS	Sitting/total height SDS	Heart rate	Blood pressure	X-ray features
Family 1 (C380fs)										
1.II.1 (MT) index	1.3	F	MG, CP, DS, DE, DW, DT, DF, CF, HC	**-2.46**	112% (1.42)	**2.29**	**5.60**[a]	80	120/70[a]	WB, TS, FP
1.I.1 (WT)	33	F	N	-0.98	29.4 (1.49)	0.33	-0.07	84	100/60	N/A
1.I.2 (WT)	37	M	N	-1.65	**32.4 (2.02)**	N/A	N/A	N/A	N/A	N/A
Family 2 (R384H)										
2.III.1 (MT) index	0.9	M	MG, CF (DW, DT, DF[c])	-0.63	106% (1.25)	**2.02**	-1.00	106	75/40	WB, TS
2.II.1 (MT)	35	F	CP, DE, DW, DT	-1.10	**30.7 (1.60)**	1.33	**3.60**	76	100/70	TS, FP
2.II.3 (WT)	32	M	N	-0.54	26.4 (0.99)	0.00	-0.39	68	120/80	N/A
2.II.2 (WT)	35	M	DE, DW, DT	-1.25	**31.3 (1.84)**	**3.20**	1.20	88	105/65	N[b]
2.I.2 (WT)	70	M	N	-0.96	**38.1 (2.52)**	**2.30**	1.00	70	120/75	TS[b]
2.I.1 (WT)	71	F	N	**-2.33**	**32.8 (1.77)**	-1.33	1.80	82	125/85	N[b]
Family 3 (A263S)										
3.III.6 (MT) index	2.6	M	CP, DE, DF	-1.18	15.9 (-0.33)	1.14	**2.71**	102	80/50	WB, TS
3.III.5 (MT)	7.4	F	N	-1.66	18.4 (1.22)	-0.11	0.29	78	100/65	WB, TS
3.III.3 (MT)	8.8	M	CP, DE, DW, DT	-1.44	17.2 (0.58)	-0.58	0.08	84	95/60	WB, TS
3.III.1 (MT)	17	F	N	-1.21	25.4 (1.11)	-0.60	0.52	84	115/75	FP[b]
3.II.3 (MT)	31	F	CP	-1.71	21.7 (0.02)	**2.00**	-0.77	68	100/60	WB, TS, FP
3.II.1 (MT)	35	F	DW, CP	-1.56	29.9 (1.52)	1.33	-0.40	86	110/70	WB, TS[b]
3.I.2 (MT)	55	M	DS, DW, DF, ST	**-2.70**	**34.3 (2.19)**	**4.00**	1.00	74	125/75	WB, TS, FP, ED
3.III.4 (WT)	10.1	F	N	-0.98	17.5 (0.27)	0.09	-1.70	76	105/60	WB
3.III.2 (WT)	11	F	N	-0.06	19.0 (0.51)	-1.20	0.55	92	115/70	N/A
3.II.4 (WT)	32	M	N	-0.41	28.6 (1.43)	0.00	-0.08	76	110/70	TS, FP
3.II.2 (WT)	40	M	N	-1.51	26.0 (0.99)	0.80	-0.42	N/A	N/A	N/A
3.I.1 (WT)	56	F	N	**-2.90**	**43.6 (2.29)**	**2.00**	-1.60	68	110/65	N[b]

N, normal; N/A, not available; MG, macroglossia; CP, constipation; DS, delayed sitting, DE, delayed tooth eruption; DW, delayed walking; DT, delayed talking; DF, delayed closure of anterior fontanelle; WB, wormian bones; TS, thickened skull; FP, frontal prominence, ST, skin tag; CF, coarse facies; HC, hoarse cry; ED, epiphyseal dysgenesis
Abnormal values are indicated in bold.

[a] Measured at the age of 12.7 years.[b] Hip X-ray could not be performed but there was no gait problem.[c]Observations were made during follow up at older age.

Table 2. Biochemical analyses in 3 families with THRA mutations

Subject N	TSH mU/L 0.4–4.3	FT4 pmol/L 11–25	T4 nmol/L 58–128	FT3 pmol/L 3.8–7.6	T3 nmol/L 1.4–2.5	rT3 nmol/L 0.22–0.52	T3/T4 x100 1.4–3.1	T3/rT3 3.1–13	Hb (Δ) g/dL [a]	CK U/L 30–168	SHBG nmol/L [b]	IGF1 ng/mL [c]	Cholesterol mg/dL < 200
Family 1 (c380fs387X)													
1.II.1 index[e] (F, 1.3y, MT)	1.4	**5.1**	**53**	**12.4**	**2.76**	N/A	N/A	N/A	**8.9 (-1.1)**	N/A	N/A	N/A	168
1.I.1[d] (F, 33y, WT)	1.17	12.0	**137**	N/A	**2.56**	0.39	1.87	6.6	12 (0.0)	59	**> 180**	224	164
1.I.2 (M, 37y, WT)	2.68	20.6	107	N/A	**2.60**	0.49	2.43	5.3	N/A	N/A	N/A	N/A	N/A
Family 2 (R384H)													
2.III.1 index (M, 0.9y, MT)	1.89	13.9	107	**8.08**	**5.20**	0.31	**4.86**	**16.8**	**8.6 (-2.4)**	**268**	125	*33.7*	199
2.II.1 (F, 35y, MT)	2.51	13.6	80	6.30	**3.32**	**0.20**	**4.15**	**16.6**	**11.2 (-0.8)**	125	70.0	*85.3*	167
2.II.3 (M, 32y, WT)	2.34	13.4	86	5.16	**2.32**	0.26	2.70	8.9	15.7 (2.7)	**193**	N/A	N/A	179
2.II.2 (M, 35y, WT)	2.51	11.5	65	5.05	2.41	0.24	**3.71**	10.0	14.1 (1.1)	139	36.2	*53.8*	167
2.I.2 (M, 70y, WT)	0.78	22.0	118	4.01	2.49	**0.55**	2.11	4.5	N/A	153	38.6	N/A	**226**
2.I.1 (F, 71y, WT)	1.99	18.6	83	4.41	1.58	0.51	1.90	3.1	13.8 (1.8)	39	20.4	N/A	157
Family 3 (A263S)													
3.III.6 index (M, 2.6y, MT)	2.10	16.4	85	7.28	**3.65**	0.31	**4.29**	11.8	11.6 (0.1)	**236**	127	*31.0*	134
3.III.5 (F, 7,4y, MT)	1.40	17.6	98	**7.96**	**3.46**	0.27	**3.53**	12.8	**10.8 (-0.7)**	**218**	93.0	78.1	154
3.III.3 (M, 8.8y, MT)	2.59	16.1	112	6.65	**2.96**	0.24	2.64	12.3	11.8 (0.3)	**240**	123	155	151
3.III.1 (F, 17y, MT)	2.03	14.4	89	6.65	**2.53**	**0.19**	2.84	**13.3**	**10.9 (-1.1)**	115	38	450	158
3.II.3 (F, 31y, MT)	0.95	16.1	87	5.94	**2.51**	0.27	2.89	9.3	**9.6 (-2.4)**	87	90.1	172	149
3.II.1 (F, 35, MT)	2.44	15.6	**131**	6.16	**3.21**	0.28	2.45	11.5	**10.5 (-1.5)**	125	78.8	168	174
3.I.2 (M, 55y, MT)	1.58	13.6	98	5.96	**2.57**	0.28	2.64	9.2	13.5 (0.5)	125	24.0	114	**215**
3.III.4 (F, 10.1y, WT)	1.44	19.5	108	6.50	**2.81**	0.34	2.60	8.3	12.0 (0.5)	115	65.7	165	172
3.III.2 (F, 11y, WT)	2.19	16.0	114	6.80	**3.14**	0.35	2.75	9.0	13.3 (1.8)	135	110	204	**208**
3.II.4 (M, 32y, WT)	1.30	19.0	100	5.30	2.05	0.47	2.05	4.4	15.9 (2.9)	119	N/A	N/A	145
3.II.2 (M, 40y, WT)	1.87	14.4	68	5.90	1.99	0.22	2.91	9.0	14.8 (1.8)	**408**	23.6	174	182
3.I.1[g] (F, 56y, WT)	< 0.015	**33.9**	**207**	6.57	**2.61**	**0.54**	**1.26**	4.8	13.3 (1.3)	72	71.0	*56*	**255**

Reference values: [a] Hemoglobin, lower limit: 0.5–2 years, 11 g/dL; 2–12 years, 11.5 g/dL; > 12 years female, 12 g/dL; male, 13 g/dL Hb levels relative to the lower normal limits are given in parentheses. In parentheses are shown the . [b] SHBG: adult, no-pregnant females, 18–144 nmol/L; adult males, 10–57 nmol/L; prepubertal males, 31–167 mmol/L; prepubertal females, 43–197 mmol/L. [c] IGF1: see reference (29); low values are indicated in italic, high values are underscored. [d] Measured during pregnancy. [e] Measured at the age of 16 months at presentation; [g] Measured on LT4 therapy for hypothyroidism.

Abnormal values are indicated in bold.

Family 1

Patient 1.II.1(index case). The index patient was born at term (birth weight 3200 g) following an eventless pregnancy. First evaluation was made at the age of 16 months due to prominent developmental delay: she could not control her head until the age of 8 months (normal milestone < 2 months[232]), at 16 months she was not able to sit (normal milestone < 8 months,[232]) and had no teeth eruption (normal milestone before 13 months ([233]). In addition, she suffered from constipation and she was noted to have a hoarse-sounding cry, coarse facies, macroglossia, and an umbilical hernia. Normocytic anemia, normal TSH, low FT4, high T3, and a normal TSH response to TRH (31.8 mU/L at 20 min) were found. At the age of 2, she was started on LT4 (4.5 µg/kg/day), and she has been treated since with various LT4 doses (1–3.7 µg/kg/day), with normalization of FT4 levels and suppression of TSH for most of the time. Despite treatment with LT4, she still had a delay in reaching several developmental milestones: sitting occurred at 26 months (normal milestone < 8 months,), resolution of umbilical hernia at 3 years, and closure of the anterior fontanelle at 5 years (normal milestone < 24 months,[234]). Menarche was at 12 years of age (normal range: 12.2 ± 0.9 years[235]). Hypertrophic obstructive cardiomyopathy, pericardial effusion, and nephrolithiasis developed during follow-up.
At her most recent visit at the age of 12.7 years, she was severely handicapped, was not able to walk, and had mild scoliosis. Her height was 127 cm (SD score [SDS] -3.49), weight 43.3 kg (SDS 0.04), body mass index (BMI) 26.85 (SDS 1.81), and head circumference 57.4 cm (SDS 3.12). Serum TSH was suppressed but, despite LT4 treatment, serum T4 and FT4 levels were at the lower limit of normal, serum rT3 was decreased, T3 and FT3 levels were increased, and the T3/T4 and T3/rT3 ratios were markedly elevated. General thickening of skull vault was demonstrated on cranial X-ray while femoral epiphyses were normal.
The mother (1.I.1) and father (1.I.2) of the index patient were not affected.

Family 2

Patient 2.III.1(index case). This boy was born after an eventless pregnancy with a birth weight of 3400 g. Macroglossia was evident at birth and was reported to ameliorate with time. He was first evaluated at the age of 8 months due to frequent upper respiratory tract infections. High FT3, low FT4 and a normocytic normochromic anemia were detected. A TRH test revealed a delayed TSH peak (24.3 mU/L) at 60 minutes. Physical examination revealed an anterior fontanelle of 5x3 cm [> 90th percentile (2.5 cm)],[234] a decreased height (-0.63 SDS), increased BMI (1.25 SDS) and head circumference (2.02 SDS). However, umbilical hernia and constipation were absent. Bone age corresponded to 6 months at the chronological age of 8 months. Lateral skull radiograph showed 3–4 wormian bones at the conjunction of lambdoid and sagittal sutures and thickening of skull at the frontal and occipital region.

At 16 months of age the boy was tested for neuromotor and neuropsychological development. His cognitive ability was only moderately impaired but his motor development was more severely impaired.

Patient 2.II.1. The 35 years old mother of the index patient also showed delay in reaching several developmental milestones (Table 1). She had her first menstruation at the age of 13 and she became pregnant naturally despite a history of irregular menses requiring treatment. In adulthood, a number of characteristics associated with hypothyroidism were present, such as constipation and a slow speech. In addition, she showed several features of RTHα, borderline intellectual functioning, impaired executive functioning and mild deficits in memory. No psychiatric pathology according to DSM-IV was evident and, the patient did not have a history of a psychiatric disorder. Her parents (2.I.1 and 2.I.2) and only sibling (2.II.2) were not affected.

Comparison of the two patients with the unaffected members indicates that elevated serum T3 and T3/rT3 ratio and anemia are distinctive features of RTHα in this family. Short stature is not a distinctive characteristic, although the affected mother (2.II.1) shows marked disproportionate growth. Head circumference and thickening of the skull are increased and serum IGF-1 is decreased in both patients but these abnormalities are also observed in the unaffected father (2.II.2) and grandfather (2.I.2) of the index patient.

Family 3

Patient 3.III.6 (index case). The index case was born at term with a birth weight of 3000 g. He was evaluated first at the age of 13 months due to frequent infections, and low FT4, normal FT3 and TSH levels were detected. He frequently suffered from constipation and had a delay in tooth eruption (normal milestone < 13 months[233] and his anterior fontanelle had closed at 2 years and 4 months (normal milestone < 24 months[234]). Macroglossia, umbilical hernia and anemia were not present. Physical examination at the age of 2 years and 7 months showed mild growth retardation with a disproportionate body habitus and a mildly increased head circumference. Skull radiographs showed thickening of the skull at the occipital region. Neurological and neuropsychological examination at the age of 2 years and 8 months yielded scores for cognition and motor development in the normal range.

The index patient has two older sisters, one of whom is also affected. The affected sister (3.III.5) showed relatively mild growth retardation but otherwise normal development. Her serum T3 levels were higher and hemoglobin was lower than in her unaffected sister. Also, her global IQ, verbal and performance scores were lower than those of her older unaffected sister (3.III.4) but they were all in the normal range.

The index patient has three cousins, two of whom are also affected. One cousin (3.III.3) had clearly more clinical features of RTHα than his oldest sister (3.III.1), including delayed developmental milestones and constipation. Like the index patient, he showed a de-

creased serum IGF1 level. He also revealed low average global, verbal and performance IQ scores. Both affected cousins had anemia.
The two mothers of the above children (3.II.1, 3.II.3) were affected. Both patients suffered from constipation and had anemia. In addition, radiological evaluation of the patients' skull X-rays showed a thickening of the skull vault in combination with wormian bones. The mother of the index patient (3.II.3) demonstrated low-average intellectual functioning, with a more prominent decrease in performance than in verbal IQ. The patient reported a period of antidepressant therapy after the suicide of her younger brother, but no psychiatric pathology was evident at the time of the interview. We were not able to evaluate any biological samples from the deceased (3.II.5), but his psychiatric story was insignificant before he committed suicide. Patient 3.II.1 had also received treatment for depression and panic attacks.
The index patient's grandfather (3.1.2) was also affected and showed several RTHα characteristics during development as well as short stature and marked macrocephaly. He displayed low-average intellectual and verbal memory and executive functioning. The patient reported a depressive episode, where he used antidepressant medication for a year, after the suicide of his son (3.II.5).
The grandparents are consanguineous. The grandmother (3.I.1) is not affected although she has long-standing hypothyroidism from an unknown cause for which she receives LT4 therapy. Remarkably, she also has a short stature and macrocephaly.
An overview comparing the clinical and biochemical characteristics in this family is presented in Figure 3. Comparing the phenotypes of the affected and unaffected members, there are remarkably few distinctive RTHα characteristic in this family. Constipation and mild developmental delays are seen in most but not all affected subjects. There appears to be no significant difference in TH levels, although the T3/rT3 ratio is higher in affected than in unaffected subjects. The most distinct feature of RTHα in this family is the low/borderline low hemoglobin level in 6 of the 7 affected subjects but in none of the unaffected relatives.

Genetic and molecular analyses

Family 1. Sequencing of genomic DNA indicated that the index patient was heterozygous for a deletion of 4 nucleotides in exon 9 of *THRA* (c.1138–1141delTGCC), resulting in a deletion and frameshift at codon 380 and an early stop at codon 387 of TRα1 (p.C380fs387X) (Figure 1). The mutation was absent in the parents of the patient, suggesting a de novo mutation in patient 1.II.1.
Family 2. Sequence analysis showed that the index patient and his mother were heterozygous for a nucleotide substitution in *THRA* (c.1151G>A), leading to an Arg to His substitution at codon 384 in TRα1 (p.R384H) (Figure 1). Remaining members of the family did not carry the mutation.

Family 3. DNA sequencing indicated that the index patient, his middle sister, mother, grandfather, maternal aunt, oldest niece and cousin were heterozygous for a nucleotide substitution in *THRA* (c.G787T), leading to an Ala to Ser substitution at codon 263 (p.A263S) common to both TRα1 and TRα2 (Figure 1). The deceased member of this family (3.II.5) was not analyzed for *THRA* mutations; remaining members were negative. All three mutations have not been detected in public databases.

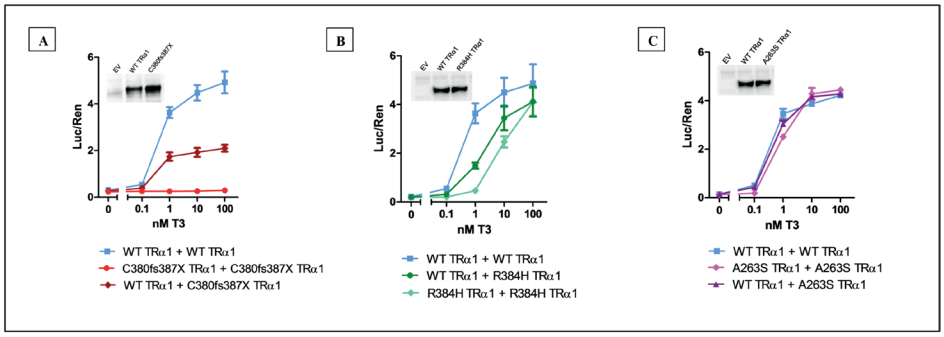


Figure 2. Functional analysis of the C380fs387X, R384H and A263S TRα1 mutants.
(A) The transcriptional activity of WT and mutant TRα1 was tested in JEG3 cells transfected with a T3-dependent promoter-reporter construct. The C380fs387X mutant had a negligible affinity for T3 compared to WT TRα1 at all T3 concentrations. When co-expressed, the C380fs387X mutant suppressed WT TRα1 function in a dominant negative manner. Results are the means ± SEM of 3–5 experiments. **(B)** At concentrations of 1–10 nM T3, the R384H mutant showed a reduced transcriptional activity, but at higher T3 levels the maximal transcriptional activity was similar to WT TRα1. Co-transfection of WT TRα1 with the R384H mutant resulted in a moderate dominant-negative effect. **(C)** The A263S mutant showed a comparable biological activity to WT TRα1, and co-expression of the A263S mutant hardly changed the function of WT TRα1.

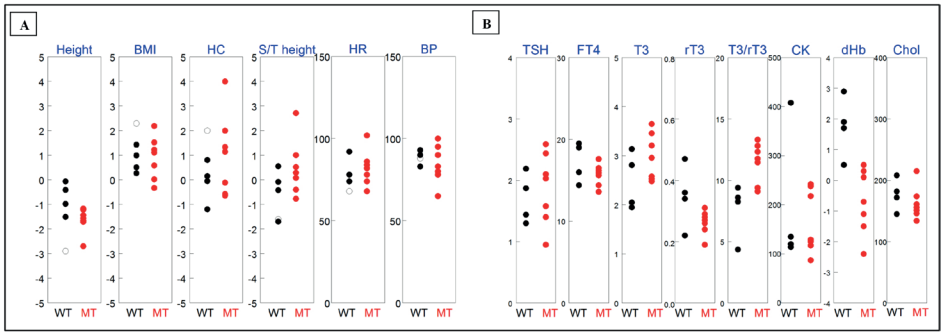


Figure 3. Clinical and biochemical parameters of family 3 (A263S TRα).
(A) The height, body mass index (BMI), head circumference (HC) and sitting-to-total height (S/T height) of both affected (MT) and unaffected (WT) individuals are expressed in SDS values. Heart rate (HR) is expressed in beats per minute and the mean arterial blood pressure (BP) is expressed in mm Hg. **(B)** TSH (mU/L), FT4 (pmol/L), T3 (nmol/L), rT3 (nmol/L), T3/rT3, CK (U/L), ΔHb (Hb vs. the lower limit of normal, g/dL) and cholesterol (mg/dL) values of both WT and MT family members are presented.

Functional analysis of the mutant receptors

To assess the effects of the mutations on the transcriptional activity of TRα1, luciferase assays were performed. In addition, the expression of all the receptor proteins was confirmed by Western blotting with an antibody against the N-terminal Flag epitope tag added to the receptor constructs. The C380fs387X, R384H and A263S mutant receptors showed similar expression levels as WT TRα1 (Figure 1).

Family 1. Analysis of JEG3 cells co-transfected with WT or mutant TRα1 and a DR+4 TRE-luciferase reporter gene showed negligible activation of the C380fs387X mutant by up to 100 nM T3 in contrast to marked activation of WT TRα1 (EC_{50} 0.4 nM T3). When co-expressed, the C380fs387X mutant suppressed WT TRα1 function in a dominant negative manner (Figure 2).

Family 2. The R384H and A263S mutations showed much milder effects on the function of TRα1. At concentrations of 1–10 nM T3, the R384H mutant TRα1 showed a reduced transcriptional activity, but at higher T3 levels the R384H mutant approached the activity of WT TRα1. EC_{50} values were 22-fold higher for the R384H mutant than for WT TRα1 (8.7nM vs 0.4 nM T3; $p < 0.05$). Co-transfection of WT TRα1 with the R384H mutant did result in a moderate dominant-negative effect (Figure 2).

Family 3. The biological activity of the A263S mutant was comparable to that of WT TRα1 at most T3 concentrations, except at the lowest concentration (0.1 nM) T3 where WT TRa1 was stimulated 3.6-fold and the A263A mutant 1.5-fold. EC_{50} values of A263S mutant and WT TRα1 differed scarcely (0.83 nM vs 0.33 nM T3; $p < 0.05$). Co-expression with the A263S mutant hardly changed the function of WT TRα1, except that 0.1 nM T3 produced an intermediate response compared with cells transfected with WT TRa1 alone or the A263S mutant alone (Figure 2). To investigate the biological activity of TRα2 and its possible interaction with TRα1, we co-transfected cells with WT TRα1 together with WT TRa2 or the A263S mutant in various ratio's (1:1–50). Independent of the T3 concentration, WT and A263S mutant TRα2 showed neither transcriptional activity nor dominant-negative activity towards WT TRα1 when overexpressed (Supplemental Figure 1).

Discussion

In the current study we describe a total of 10 RTHα patients with three different mutations in TRα. This large case series enables us to report on the diversity in the phenotype of patients with different mutations and compare the phenotypes of both affected and non-affected individuals from a family with the same mutation.

So far, 4 patients from 3 families have been described with frame-shift mutations in the C-terminal domain of TRα1: the F397fs406X mutation found in two patients from the

same family by van Mullem *et al.*,[130,153] the A382fs388X mutation found in one patient by Moran *et al.*,[188] and the C380fs387X mutation in patient 1.II.1 in the present study. Although only 3 frameshift mutations have been identified yet, the more upstream the shift of these mutations occurs, the more severe the clinical phenotype appears to become. The father and daughter with the most downstream F397fs406X mutation showed marked short stature and mild delay of motor and mental development but had normal IQ.[130,153] They had been treated with LT4 from the age of 6 and 42 years, respectively. The 45-year-old female with the A382fs388X mutation, who had been treated with LT4 from the age of 3 months, presented with more severe cognitive impairment and constipation, but she was still able to walk and talk, and had only mild short stature.[188] Patient 1.II.1 with the most upstream C380fs387X mutation has a more severe phenotype than the above cases. Late initiation of LT4 treatment at 2.5 years of age cannot be the only cause for her global developmental delay as LT4 treatment of the patients with the F397fs406X mutation was started even much later.[130,153] Also growth did not ameliorate with LT4 treatment, and cardiac and renal problems that may reflect tissue hypothyroidism developed during treatment. All three frame-shift mutations result in a clear dominant-negative effect on WT TRα1 in *in vitro* assays, explaining the marked pathogenic activity of these heterozygous mutations in the patients. At present it is unknown if this dominant-negative activity increases with the more upstream location of the frame-shift mutation.

So far, 3 nonsense *THRA* mutations have been reported. Bochukova *et al.*[151] found an E403X mutation in a 6-year-old girl with developmental delay, clumsiness, severe constipation, disproportionate short stature, macrocephaly, and femoral epiphyseal dysgenesis. Tylki-Szymanska *et al.*[220] reported the same E403X mutation in a 15-year-old female who demonstrated similar features without femoral epiphyseal dysgenesis. Similar to the patients with frame-shift *THRA* mutations, they also found a more upstream mutation (C392X) leading to a more severe phenotype,.[220] There were no nonsense mutations in our case series.

Different missense *THRA* mutations have previously been reported in RTHα patients with milder clinical phenotypes,[219–221] and such mutations were also identified in families 2 and 3 in the present study. We report for the first time that spontaneous conception and eventless pregnancy can occur in untreated females with RTHα (2.II.1, 3.II.1 and, 3.II.3) despite varying signs of hypothyroidism. Furthermore, it is noteworthy that case 2.II.1 had a similar and even more remarkable delay in developmental milestones compared to her affected son (patient 2.III.1). However, as an adult, she had a milder intellectual deficit, no cardiac problems, and normal FT3, FT4, and TSH levels, suggesting amelioration of the clinical phenotype with time. Similar observations were made in a mouse model with a heterozygous TRα1 mutation at the same position as in family 2, i.e. R384C.[111,207] These mice showed severe but transient impairment of post-

natal development and growth. Alterations in TH levels were also only evident during the juvenile period. The mechanisms underlying the amelioration of deficits caused by TRα1 mutations with age are unknown.

The third family is the largest pedigree with RTHα reported so far, and includes patients with the mildest phenotype in terms of growth, body habitus, and cognitive functions as well as the oldest untreated patient (3.I.2). This large family allowed us to observe the heterogeneous clinical consequences of the same THRA mutation in a single family, which is very similar to what has been described for RTHβ.[7,236] Both affected and non-affected family members showed appreciable variation in the clinical and biochemical characteristics (Figure 3). Overall, patients with the A263S TRα1 mutation differed from healthy individuals by a higher serum T3/rT3 ratio and lower hemoglobin levels. Affected individuals also displayed a shorter body stature in combination with a larger head circumference, although there was a large overlap with the unaffected subjects.

The A263S mutation in this family is located at the same location as the A263V mutation found recently in a 60-year-old woman and her 2 young adult sons with RTHα.[219] The A263V mutation resulted in poor linear growth, delayed developmental milestones, constipation, and macroglossia during infancy, and they had been treated with LT4 since 2–3 years of age.[219] Their final height and body habitus were normal but all had increased head circumference. Two of them had numerous skin tags while only one skin tag was present in a single individual in our series (patient 3.I.2). The functional analysis of the A263V mutant also indicated a greater defect in transcriptional activity of TRα1 than that induced by the A263S mutation,[219] in further support of some degree of genotype–phenotype correlation for *THRA* mutations.

The Ala263 mutations are present in both TRα1 and TRα2. In line with previous studies of patients with the A263V or D211G (also affecting TRα1 and TRα2) mutation, we did not find any evidence for additional abnormalities caused by consequences of the mutation on TRα2.[219,221] In vitro studies did not provide any evidence for a dominant-negative effect of WT or mutant TRα2 over TRα1, in agreement with findings that the TRα2 protein is located in the cytoplasm and thus cannot interfere with the interaction of TRα1 with genomic elements and other nuclear factors.[237,238]

The A263S mutation in this family and the A263V mutation mentioned above[219] are present at the corresponding position as a mutation in *THRB* (A317V) recently identified in a family with RTHβ.[239] It is also of interest to note that the R384H and R384C mutations have been identified repeatedly at the corresponding positions R438H and R438C, respectively, in *THRB* in patients with RTHβ,[240] suggesting that this is also a hot spot for mutations in RTHα. As in RTHβ, the identified mutations in TRα are mostly located within three CpG-rich hot spots in the ligand-binding domain of the receptor. Considering the strong homology between the TRα1 and TRβ1 receptors and the

comparable localization pattern of mutations in both receptors, the incidence of RTHα could be similar to that of RTHβ (1:40,000).[7]

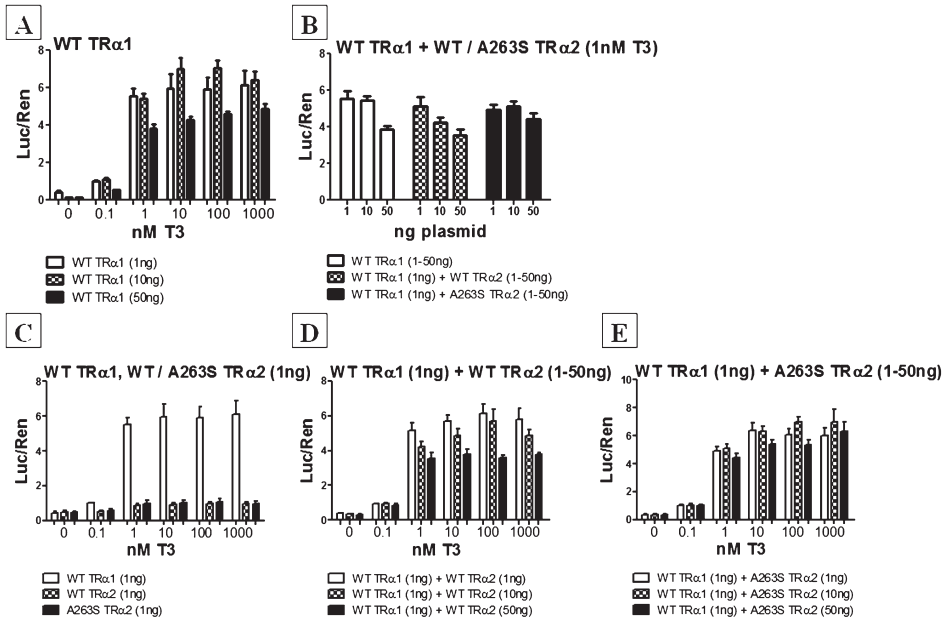
Despite T3 resistance at the receptor level, (F)T3 levels are not always high. In all previously reported RTHα patients, (F)T4 levels were low or at the lower limit of normal. In the current study, we observed that (F)T4 levels were initially low in index cases initially but increased into the normal range during follow-up. Accordingly, none of the affected adults had low serum (F)T4, suggesting attenuation in time. If rT3 levels were measured in previous cases, they were all low which may be explained by lower precursor (F)T4 levels and down-regulation of type 3 deiodinase activity. In the present study, pretreatment rT3 levels were in the normal range except for two moderately affected patients (2.II.1, 3.III.1). TSH levels were normal in all patients with *THRA* mutations, including our cases, except for the 45-year-old female patient reported by Moran *et al*., who showed a slightly increased TSH of 5.8 mU/L (N 0.35–5.5) after stopping LT4 following long-term treatment.

Despite the fact that (usually normocytic normochromic) anemia is not present in all RTHα patients, it seems to be a frequently appearing characteristic. Several studies in humans have indicated an association between hypothyroidism and anemia.[201,202] In addition, data from animal models point towards an important role for TRα in erythropoiesis.[203–206] This supports the hypothesis that mutations in TRα contribute to the pathogenesis of anemia seen in RTHα patients.

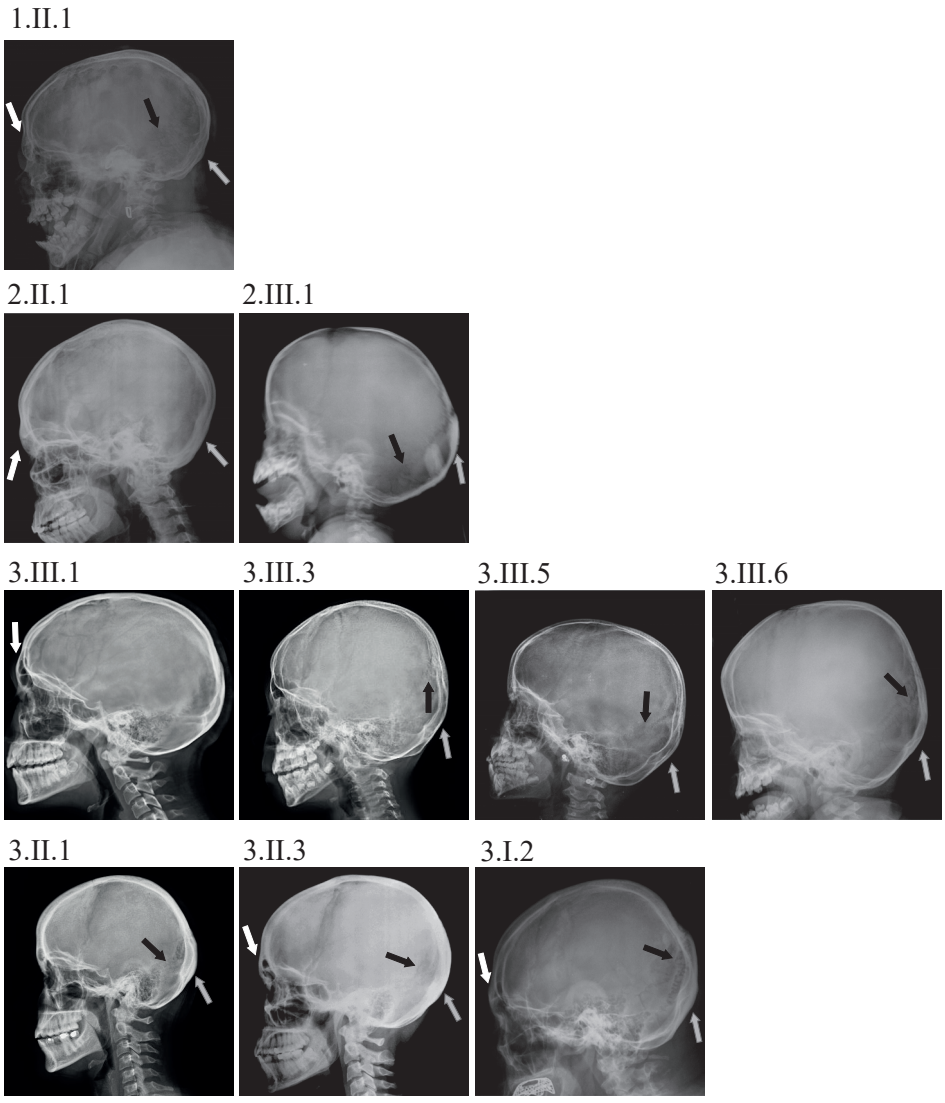
In conclusion, this study shows that the clinical consequences of mutations in *THRA* are heterogeneous. This is the case for patients with different TRα mutations, but also for family members with the same mutation. Common characteristics in almost all patients comprise a delay in reaching developmental milestones, constipation, and anemia. Growth retardation and macrocephaly are less consistent. The clinical phenotype varies from severe to very mild, in part depending on the type and location of the mutation. Therefore, mutations in *THRA* should be suspected in subjects with even mild developmental delays, anemia, and a high serum T3/rT3 ratio.

Acknowledgements

We are grateful to the families for their contribution. We also thank Berge Velibaşoğlu, MSc for her help in performing neuropsychological assessments and interpreting the results and Assoc. Prof.dr. Serkan Yener for his kind support in hormone assessments of some adult subjects.



Supplemental Figure 1. Functional analysis of WT and A263S mutant TRα2.
(A) JEG3 cells were transfected with a T3-dependent promoter-reporter construct and increasing amounts of WT TRα1 (1–50 ng plasmid). Higher plasmid concentrations (≤ 10 ng) resulted in a decreasing transcriptional activity. **(B)** Co-transfection of WT TRα1 with increasing amounts of WT or A263S mutant TRα2 (1–50 ng) resulted in a decreased transcriptional activity, comparable to the effect of increasing amounts of WT TRα1 (1–50 ng) alone. **(C)** JEG3 cells were transfected with WT TRα1, WT TRα2 or A263S mutant TRα2 (1 ng) and incubated with increasing T3 concentrations (1–1000 nM). At all T3 concentrations, WT TRα2 and A263S mutant TRα2 showed negligible transcriptional activity. **(D)** and **(E)** Co-transfection of WT TRα1 (1 ng) with increasing amounts WT or A263S mutant TRα2 (1–50 ng) produced mildly reduced transcriptional activity, similar to the effect of increasing the WT TRα1 concentration (1–50 ng) alone (panel B).



Supplemental Figure 2. Radiological investigation of the patients.
Skull X-rays of the patients demonstrating features noted in Table 1. White arrows indicate frontal prominence, black arrows wormian bones, and grey arrows thickened skull vault.

Supplementary Table 1. Primers used for PCR amplification and sequencing of THRA

Exon	Forward primer (5′-3′)	Reverse primer (5′-3′)
6	TTCTCCAACCTGTACTCTAGGAAGA	TCCTGGAGGAGGCAAGACT
7	CTTGGAGCTCCCCCTGGT	TCCTTGTCCAGAGAACCTCAG
8	GGCTCCCGTAGGACACTCTA	ATTCAGGAGGGAGTTGAGCA
9	TCCCCTCTAGTCCTTTCTTCC	TGTGTGTGTGGGAGCTGAAT

Supplementary Table 2. Neuropsychological and neuromotor development of affected and unaffected individuals tested.

Case	Test	Score	Corresponding age	Classification
2.III.1 index (M, 16mo, R384H)	Cognition	82	14 mo	Moderately impaired
	Motor development	57	10 mo	Severely impaired
2.II.1 (F, 35y, R384H)	Global IQ	75		Borderline
	Verbal IQ	77		Borderline
	Performance IQ	74		Borderline
3.III.6 index (M, 32mo, A263S)	Cognition	85	27 mo	Average
	Motor development	94	30 mo	Average
3.III.5 (F, 7.4y, A263S)	Global IQ	91		Average
	Verbal IQ	92		Average
	Performance IQ	92		Average
3.III.4 (F, 10.1y, WT)	Global IQ	104		Average
	Verbal IQ	98		Average
	Performance IQ	108		Average
3.III.3 (M, 8.8y, A263S)	Global IQ	83		Low average
	Verbal IQ	89		Low average
	Performance IQ	72		Borderline
3.II.3 (F, 31y, A263S)	Global IQ	85		Low average
	Verbal IQ	93		Average
	Performance IQ	76		Borderline
3.I.2 (M, 55y, A263S)	Global IQ	89		Low average
	Verbal memory	84		Low average
	Executive functions	86		Low average

ANEMIA IN PATIENTS WITH RESISTANCE TO THYROID HORMONE ALPHA (RTHα): A ROLE FOR TRα IN HUMAN ERYTHROPOIESIS

7

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Journal of Clinical Endocrinology and Metabolism 2017

Abstract

Context

Patients with Resistance to Thyroid Hormone alpha (RTHα) are characterized by varying degrees of growth retardation, macrocephaly, constipation, and abnormal thyroid function tests. In addition, almost all RTHα patients have mild anemia, the pathogenesis of which is unknown. Animal studies suggest an important role for TH and TRα in erythropoiesis.

Objective

Our objective was to investigate whether a defect in TRα affects the maturation of red blood cells in RTHα patients**.**

Design, setting and patients

Cultures of primary human erythroid progenitors (HEPs), from peripheral blood of RTHα patients (n = 11) harboring different inactivating mutations in TRα (P398R, F397fs406X, C392X, R384H, A382fs388X, A263V, A263S) were established and compared to healthy controls (n = 11). During differentiation, erythroid cells become smaller, accumulate hemoglobin, and express different cell surface markers. We therefore assessed cell number and cell size, and used cell staining and FACS analysis to monitor maturation at different time points.

Results

After ~14 days of *ex vivo* expansion, both control and patient-derived progenitors differentiated spontaneously. However, RTHα-derived cells differentiated more slowly. During spontaneous differentiation, RTHα-derived HEPs were larger, more positive for c-Kit (a proliferation marker), and less positive for GPA (a differentiation marker). The degree of abnormal spontaneous maturation of RTHα-derived progenitors did not correlate with severity of underlying TRα defect. Both control and RTHα-derived progenitors responded similarly when differentiation was induced. T3 exposure accelerated differentiation of both control and RTHα patient-derived HEPs.

Conclusions

Inactivating mutations in human TRα affect the balance between proliferation and differentiation of progenitor cells during erythropoiesis, which may contribute to the mild anemia in most RTHα patients.

Introduction

Erythropoiesis is the process that involves the maturation of hematopoietic progenitor cells to differentiated red blood cells (erythrocytes). Erythrocytes are of fundamental importance for all vertebrates, since they provide cells with oxygen in exchange for carbon dioxide.[241,242] The site of erythropoiesis changes throughout human development. During early embryonic development, erythropoiesis occurs in the yolk sac. By the 3rd-4th month of gestation this primitive function is taken over by the liver. From the 7th month of gestation onwards and throughout adulthood, the bone marrow is the predominant erythropoietic organ.[243–246]

The production of sufficient numbers of mature red blood cells requires a fine balance between proliferation and differentiation of progenitor cells. The cytokine erythropoietin (Epo) plays a key role in this process, along with other growth factors such as interleukin 3 (IL3), stem cell factor (SCF), and insulin-like growth factor I (IGF-I).[242,247,248]

In addition, thyroid hormone (TH) is also important for erythropoiesis. Patients with hypothyroidism frequently have anemia[201] and mice with congenital primary hypothyroidism are also anemic.[249] The action of TH is mediated via binding of the active ligand (T3) to nuclear TH receptors (TRs), TRα and TRβ**.** TRs are ligand-inducible transcription factors that regulate target gene expression by binding to TH response elements (TREs) in promoters of T3-responsive genes.[7,105,106]

Interestingly, the presence of v-ErbA, an oncogenic homologue of TRα, disturbs the balance between proliferation and differentiation of immature avian erythrocytes during erythropoiesis, contributing to fatal erythroleukemia.[250–252] Additional evidence for the involvement of T3 and TRα in erythropoiesis is provided by observations in mice lacking TRα (TRα$^{-/-}$), showing compromised fetal and adult erythropoiesis with a decreased number of erythroid progenitors in TRα$^{-/-}$ fetal livers and impaired transit of TRα$^{-/-}$ erythroblasts through further stages of maturation.[204] Other studies with TRα knockout mice, displaying defective spleen erythropoiesis, confirm that T3 via TRα stimulates late steps of erythroid development.[203]

In 2012, the first patients with resistance to thyroid hormone alpha (RTHα) due to inactivating mutations in *THRA* were discovered. All patients identified since then have monoallelic mutations in the ligand-binding domain of TRα. The phenotype of RTHα patients is characterized by growth restriction, varying degrees of neurodevelopmental retardation, macrocephaly, constipation, and abnormal thyroid function tests (low/low-normal FT4 and high/high-normal T3 levels with a normal TSH).[107,130,151,153,188,189,192,193,221,253] In addition, a mild, usually normochromic and normocytic, anemia is a virtually universal finding in RTHα patients. However, in three cases the mean corpuscular volume was raised.[151,188,193]

Given the observations that most RTHα patients have anemia and that aberrant TRα signaling affects erythropoiesis in animal models, we hypothesized that mutations in TRα affect the balance between proliferation and differentiation in the later stages of human erythropoiesis.

Patients, materials and methods

Cells and cell culture

Peripheral blood (5–10 mL) was obtained by venesection and collected into heparin or EDTA tubes from 11 RTHα patients, who have been described previously,[153,188,189,192,253] and 11 healthy donors (n = 3 related and n = 8 non-related). The study was approved by the Medical Ethics Committee of the Erasmus Medical Center. Written informed consent was obtained from all subjects and/or their parents. Mononuclear cells (PBMC) were purified from peripheral blood by density gradient centrifugation using Ficoll (Axis-Schield, Oslo, Norway).

Human erythroid progenitor cells (HEPs) were expanded in StemSpan™ SFEM medium (Stem Cell Technologies, Grenoble, France) supplemented with lipids (40 µg/mL cholesterol-rich lipid mix; Sigma), penicillin-streptomycin (1:100, Lonza, Basel, Switzerland), recombinant human erythropoietin (Epo, 2 U/mL, Janssen-Cilag, Baar, Switzerland), recombinant human Stem Cell Factor (SCF, 100 ng/ml, R&D Systems, Minneapolis, MN, USA), human IL-3 (1 ng/ml, R&D systems), human IGF-1 (40 ng/ml, R&D systems), and dexamethasone (Dex, 1 µM, Sigma, St. Louis, MO, USA).[254,255] After 4–5 days, HEPs were purified by density purification (Percoll, GE Healthcare, Little Chalfont, UK), and further expanded in StemSpan containing Epo, SCF, and Dex.

After sufficient expansion (10–15 days), 10 nM T3 (Sigma) was added to part of the HEPs, cultured under proliferation conditions. To induce differentiation, HEPs were washed 3 times with PBS (Lonza) and switched to StemSpan medium containing Epo (10 U/mL), human serum (3%, Sigma), and iron-saturated transferrin (1:100, Scipac, Kent, UK).

HEPs were maintained at 1–1.5 x10^6 cells/mL during proliferation and at 1.5–2 x10^6 cells/mL during differentiation by daily partial medium changes. To confirm that these cells are a good TRα-expressing model, RNA was extracted using TRI Reagent (Sigma) and deep sequencing was performed (using the Illumina Hiseq2500 platform (single read43bp)) in a subset of control HEPs during late proliferation (Supplemental Figure 1).

Cell count and morphology

The number of HEPs was daily monitored by cell count (CASY® INNOVATIS TTC Cell Counter, Omni Life Science, Raynham, MA, USA). To analyze cell morphology at various stages of proliferation and differentiation, 10^5 HEPs were centrifuged onto glass slides,

and stained with Benzidine (Sigma) to detect hemoglobin-expressing cells, and with Diff-Quick staining (Medion Diagnostics, Miami, FL, USA). Images were made using an Olympus BX40 microscope (40x objective, NA 0.65, Olympus, America Inc., Valley, PA, USA) equipped with an Olympus DP50 CCD camera (Olympus, America Inc., Valley, PA, USA) and Viewfinder lite 1.0 acquisition software (Better light, Inc., San. Carlos, CA, USA). The images were processed using Adobe Photoshop SC6 (Adobe, San Jose, CA, USA).

Flow cytometry

HEPs (3 x10^5 cells) were briefly centrifuged after which the pellet was resuspended in PBS supplemented with 0.1% bovine serum albumin and 1 mM EDTA (FACS buffer) and stained for 30 minutes at RT with fluorescently labeled antibodies against the following cell surface markers (all BD Pharmingen antibodies, San Diego, CA, USA): CD117 (stem cell factor receptor c-Kit, dilution 1:100), CD71 (transferrin receptor, dilution 1:100) and CD235a (glycophorin A, GPA, dilution 1:1000) in a final volume of 100 µl. To exclude non-viable cells, 7-AAD (A1310, Thermo Fisher Scientific, Waltham, MA, USA, dilution 1:1200) was added to all samples. Fluorescence was measured on a FACS Fortessa instrument (BD Biosciences, Oxford, UK) and data were analyzed using FlowJo v.10.1 software (FlowJo, Ashland, OR, USA).

Results

Genotypic and phenotypic characterization of RTHα patients

All 11 patients were heterozygous for a mutation in the C-terminal, ligand-binding domain of TRα1. In patients 5 to 9 (P5–9) the mutation (A263S/V) also affected TRα2. When studied *in vitro*, the mutant receptors showed defective hormone-dependent activation and inhibited WT TRα1 function in a dominant negative manner when co-expressed.[130,188] The severity of mutant receptor dysfunction varied depending on the type of the mutation. Patients 1 to 3 (P1-P3) have frameshift/premature stop and P4 a nonsense mutation, all generating a truncated receptor with negligible T3-induced transcriptional activity and marked dominant-negative activity. In contrast, P5–11 have missense mutations that cause reduced T3 sensitivity.

All RTHα patients with a severe mutation (P1 to P4), as well as most patients with milder mutations (P5 to P7 and P9 to P11), exhibited mild anemia. There was no correlation between the severity of the mutations and hemoglobin levels or red blood cell count (Table 1). Except for P4, P9 and P11, RTHα patients were treated with levothyroxine (LT4).

Table 1. Hematological data of RTHα patients.

Subject	Mutation	Sex	Age	LT4	Hb	Ht	RBC	MCV	MCH	Reticulo-cytes	Platelets	WBC
P1	F397fs406X	F	16	Yes	11.1 (12.5–16.0 g/dL)	0.34 (0.37–0.47 L/L)	3.95 (4.2–5.4 $*10^{12}$/L)	86.6 (78–100 fL)	28.1 (27–31 pg)	0.9 (0–2%)	235 (150–400 $*10^{9}$/L)	9.07 (4.0–11.0 $*10^{9}$/L)
P2	F397fs406X	M	52	Yes	13.3 (13.5–18.0 g/dL)	0.38 (0.42–0.52 L/L)	4.20 (4.3–5.9 $*10^{12}$/L)	89.5 (78–100 fL)	31.1 (27–31 pg)	0.89 (0–2%)	203 (150–400 $*10^{9}$/L)	6.47 (4.0–11.0 $*10^{9}$/L)
P3	A382PfsX7	F	48	Yes	12.0 (11.5–16.0 g/dL)	0.36 (0.35–0.46 L/L)	3.42 (3.8-.5.3 $*10^{12}$/L)	104 (80–100 fL)	35.1 (27–32 pg)	0.67 (0.32–2.5%)	95 (150–400 $*10^{9}$/L)	4.80 (4.0–11.0 $*10^{9}$/L)
P4	C392X	M	20	No	9.3 (12.0–16.5 g/dL)	28.4 (0.36–0.50 L/L)	3.12 (4.0–5.5 $*10^{12}$/L)	91.1 (80–100 fL)	29.7 (26–34 pg)		154 (150–400 $*10^{9}$/L)	4.6 (4.0–10.0 $*10^{9}$/L)
P5	A263S	M	4	Yes	11.0 (11.0–16.0 g/dL)	0.32 (0.35–0.48 L/L)	3.88 (4.0-.6.0 $*10^{12}$/L)	84.4 (80–100 fL)	28.2 (28–32 pg)	0.47 (0.5–1.5%)	255 (150–400 $*10^{9}$/L)	8.97 (4.5–10.5 $*10^{9}$/L)
P6	A263S	F	7	Yes	10.8 (11.0–16.0 g/dL)	0.32 (0.35–0.48 L/L)	3.91 (4.0-.6.0 $*10^{12}$/L)	83.6 (80–100 fL)	27.6 (28–32 pg)	0.51 (0.5–1.5%)	268 (150–400 $*10^{9}$/L)	4.38 (4.5–10.5 $*10^{9}$/L)
P7	A263S	F	31	Yes	9.6 (11.0–16.0 g/dL)	0.31 (0.35–0.48 L/L)	3.80 (4.0-.6.0 $*10^{12}$/L)	82.0 (80–100 fL)	25.2 (28–32 pg)	0.43 (0.5–1.5%)	206 (150–400 $*10^{9}$/L)	4.02 (4.5–10.5 $*10^{9}$/L)
P8	A263S	M	55	Yes	13.5 (13.5–17.5 g/dL)	0.40 (0.41–0.53 L/L)	4.34 (4.0-.6.0 $*10^{12}$/L)	94.4 (80–100 fL)	31.2 (28–32 pg)	1.49 (0.5–1.5%)	249 (150–400 $*10^{9}$/L)	5.18 (4.5–10.5 $*10^{9}$/L)
P9	A263V	M	17	No	11.9 (13.0–17.0 g/dL)	0.36 (0.37–0.49 L/L)	3.79 (4.5-.5.3 $*10^{12}$/L)	94.4 (78–100 fL)	31.5 (28–32 pg)	58.9 (20–120 $*10^{9}$/L)	170 (150–400 $*10^{9}$/L)	5.9 (4.0–11.0 $*10^{9}$/L)
P10	R384H	F	35	Yes	10.7 (11.0–16.0 g/dL)	0.34 (0.35–0.48 L/L)	3.91 (4.0–6.0 $*10^{12}$/L)	86.1 (78–100 fL)	27.2 (28–32 pg)	1.60 (0.5–1.5%)	241 (150–400 $*10^{9}$/L)	4.90 (4.5–10.5 $*10^{9}$/L)
P11	P398R	F	8	No								

Since blood samples were obtained from anonymous healthy blood donors (3 females, 8 males, age range 18–61), exact hematological data were not available. However, none of the donors had anemia since hemoglobin levels are required to be within the reference range (12.5 – 17.5 g/dL in female donors and 13.5 – 19.0 g/dL in male donors of Sanquin Blood Bank).

Delayed spontaneous differentiation in HEPs of RTHα patients

Peripheral blood mononuclear cells were isolated from RTHα patients and healthy controls, and cultured in conditions permissive for proliferation. The population of expanding human erythroid progenitors (HEPs) was purified by Percoll density centrifugation after 4–5 days, depending on the number of cycling progenitor cells at day 0. Once homogenous HEP populations were established (after 10–15 days), cultures were monitored daily for cell number and cell size.

After approximately 2 weeks of proliferation, normal HEPs start to differentiate spontaneously, a process characterized by a decrease in proliferation rate, reduced cell size, cytoplasmic acidification, hemoglobin production, and nuclear condensation followed by enucleation.[256] This reduction in growth rate and cell size was delayed in the RTHα cells, suggesting reduced spontaneous differentiation capacity in these cells. This was further studied by Benzidine/Diff-Quick staining of cytospin preparations. Proliferating, hemoglobin-negative, HEPs appear as large cells with blue cytoplasm, while differentiating, hemoglobin-positive HEPs are smaller with brown cytoplasm[203] (Figure 1 flowchart[254]). Consistent with our hypothesis, at a late phase of proliferation (culture day ~ 14), cytospin preparations from control cultures (n = 11), showed a substantial number of cells with differentiated morphology, marked by smaller and dark-stained cells. In contrast, preparations from RTHα patients (n = 11) showed predominantly large light-stained cells, indicating that most cells had not yet entered the differentiation program (Figure 2A).

The difference in cell size was confirmed by flow cytometry analysis. To distinguish large cells from small cells a cut-off of ≥ 100K was used, measured with forward scatter (FSC-A). The forward scatter (FSC-A) showed that cells from RTHα patients (n = 11) had a larger mean size compared to cells of controls (n = 11) (41.7% of cells in patients vs

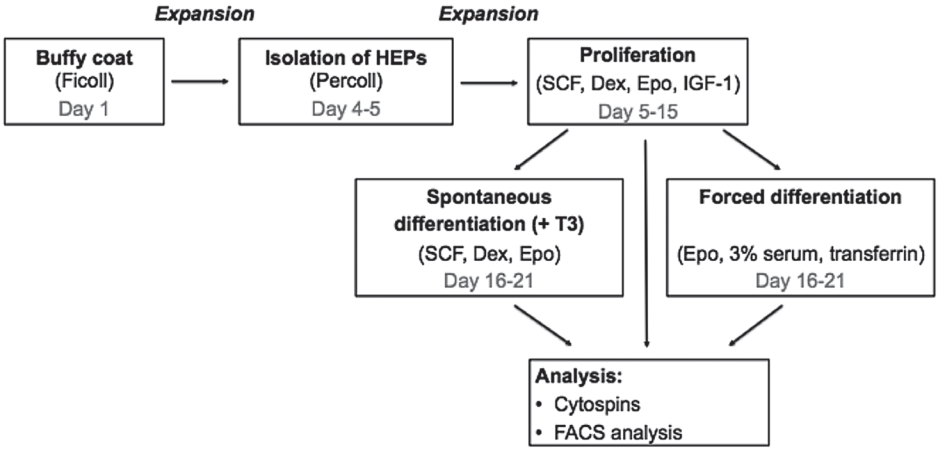


Figure 1. Flowchart of methods.

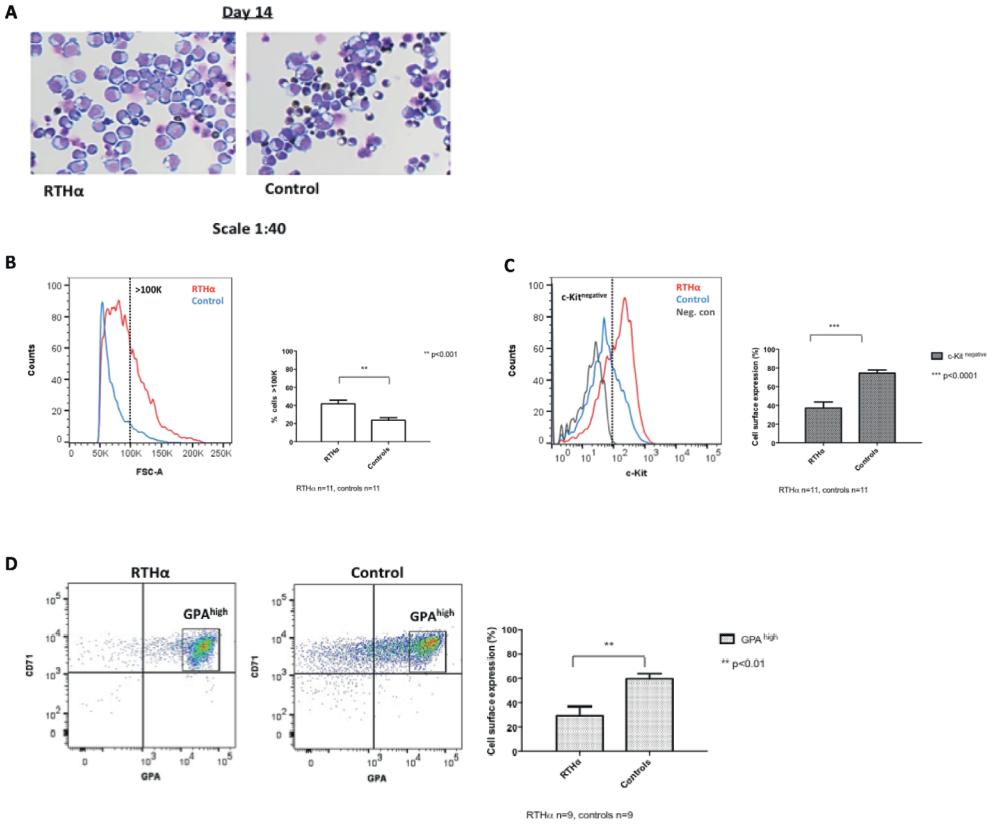


Figure 2. Spontaneous differentiation is delayed in HEPs from RTHα patients.
(A) Benzidine and Diff-Quick stained cytospins of RTHα-derived (P4; C392) and control HEPs. After 2 weeks of proliferation (day 14), control HEPs start to differentiate spontaneously, illustrated by smaller and dark-stained (hemoglobin containing) cells. In contrast, cytospin preparations from RTHα-derived HEPs show predominantly large, weakly stained cells that are still proliferating.
(B) FACS analysis of RTHα-derived and control HEPs after 2 weeks of proliferation (day 14). RTHα-derived HEPs had a larger mean size compared to control HEPs, illustrated by the percentage of cells with a forward scatter index large than 100K (41.7% of cells in patients vs 23.6% in controls, $p < 0.001$).
(C) FACS analysis of RTHα-derived and control HEPs during late proliferation (culture day ~ 14). RTHα-derived HEPs express predominantly c-Kit, a proliferation cell surface marker (37.2% of cells in patients vs 74.5% of cells in controls accumulate in the c-Kit^{negative} –subgate, $p < 0.0001$), while the majority of control HEPs express differentiation cell surface markers (CD71 and GPA) (29.6% of cells in patients vs 59.7% of cells in controls accumulate in the GPA^{high} -subgate, $p < 0.01$) **(D)**.

23.6% in controls had a forward scatter index larger than 100K, $p = 0.001$), indicating that many RTHα HEPs were still expanding, whereas the majority of control HEPs had started their differentiation program (Figure 2B).

HEPs express stage-specific cell surface proteins during different phases of erythropoiesis. CD-117 (c-Kit) is a marker of immature erythroblasts, CD71 (transferrin receptor)

expression increases during early differentiation, while Glycophorin A (GPA; CD235a) is a marker for mature erythroid cells.[254,257] During the later stages of proliferation (culture day ~ 14), control HEPs (n = 11) were predominantly negative for c-Kit as illustrated by an accumulation of HEPs in the c-Kit^{negative} -subgate (Figure 2C). However, RTHα HEPs (n = 11) accumulated less in the c-Kit^{negative} -subgate (37.2% in patients vs 74.5% in controls, $p < 0.0001$), corresponding to a delayed maturation stage. In addition, RTHα patients (n = 9, due to insufficient cells in 2 samples for GPA staining) showed less HEPs in the GPA^{high} -subgate compared to controls (n = 9) (29.6% in patients vs 59.7% in controls, $p = 0.003$), again indicating that RTHα HEPs display a reduced or delayed spontaneous differentiation rate compared to control HEPs (Figure 2D).

Despite similar culture conditions, the period in which cells could be maintained in culture varied slightly between different experiments. Nevertheless, for all experiments, the longer the cells were cultured, the more cells differentiated fully and then became apoptotic, reducing availability of cell samples for experiments focusing on later stages of maturation. No differences in the extent of apoptosis between patient and control HEPs were observed. Patient cells (n = 7) and control samples (n = 5) were further monitored during culture days ~15 to 20, still under proliferative conditions. The first signs of differentiation of RTHα cells were observed around day 16, ~2 days later than control cells. At this time point, the majority of the cells was reduced in size, but did not stain for hemoglobin (Figure 3A, top panel). After approximately 20 days of culture, apoptosis occurred in fully differentiated cells from most controls and RTHα patients, with no differences between cells with either frameshift/stop or missense mutations. Longer term observations were made with the samples that still contained viable cells. After ~20 days, the culture of control cells (n = 2) was terminated, as these cells were fully differentiated and started to disintegrate. In contrast, most RTHα cultures (n = 3) contained 2 viable subpopulations: differentiated, hemoglobin-positive cells, and immature blasts (Figure 3A, lower panel). Although the number/percentage of blasts varied per patient/mutation, these immature cells could be maintained for an additional 5–7 days, followed by apoptosis. The differences in cell size between RTHα and control cultures during the different stages is shown in Figure 3B.

The delay in spontaneous differentiation of RTHα cells was also confirmed by FACS analysis. During differentiation, a large proportion of RTHα HEPs became negative for c-Kit and simultaneously increased the expression of GPA. Values for c-Kit and GPA expression in RTHα-derived cells reached levels comparable to controls, but with a delay of 4 days (Supplemental Figure 2). During this period, expression values of the different cell surface markers in the control cells did not change, as maximal levels were already reached.

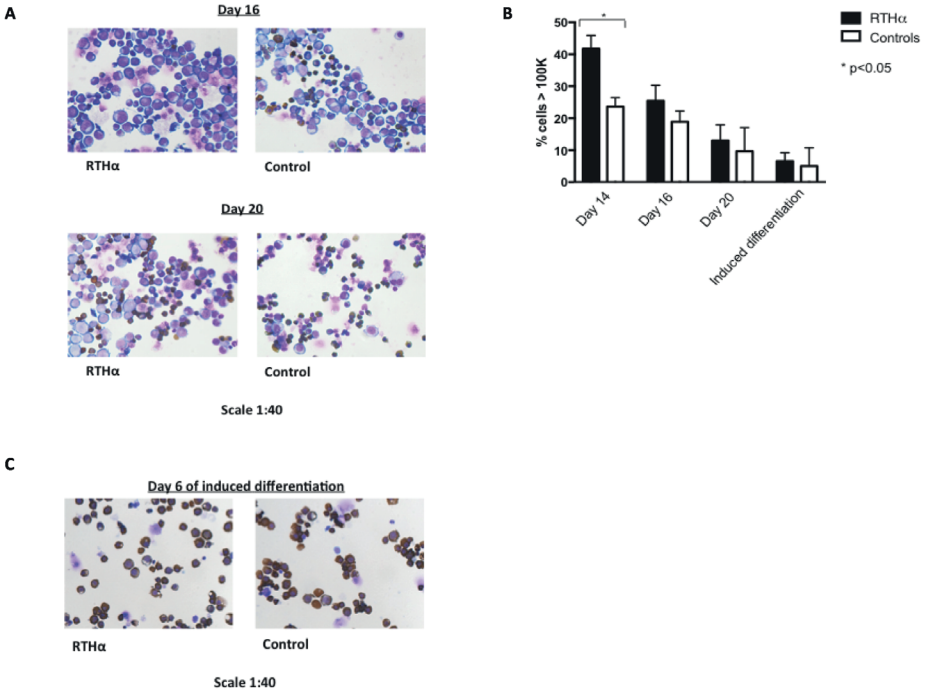


Figure 3. Differences between differentiating RTHα-derived and control HEPs decrease wherein RTHα-derived HEPs remain just behind control HEPs.
(A) Cytospin preparations of RTHα-derived (P5; A263S) and control HEPs at day 16 (early spontaneous differentiation) and day 20 (late spontaneous differentiation).
(B) Differences in cell size between RTHα-derived and control cultures during the different stages of differentiation, measured by flow cytometry analysis using forward scatter (FSC-A). To distinguish large cells from small cells a cut-off of ≥ 100K was used.
(C) Cytospin preparations of RTHα-derived (P1; F397fs406X) and control HEPs after 6 days of induced differentiation, in which HEPs were cultured in StemSpan medium containing a high concentration of Epo, human serum and, transferrin.

Taken together, these results show that onset of spontaneous differentiation is delayed in RTHα HEPs. However, when RTHα cells finally differentiate, differences between RTHα and control cells diminish, albeit with RTHα HEPs lagging behind control HEPs.

Induced differentiation proceeds similarly in RTHα and control HEPs

Optimal and synchronous differentiation of HEP cells can be induced by changing the culture conditions. To study optimal or 'forced' differentiation, HEPs were switched to StemSpan medium containing a high concentration of Epo (10 U/mL), human serum, and transferrin, whilst the proliferation factors (SCF and dexamethasone) were removed. After 6 days of induced differentiation, cell populations of the remaining RTHα patients (n = 3) and controls (n = 2) predominantly consisted of small, dark-stained and enucle-

ated cells (Figure 3C). The expression of the different cell surface markers did not differ significantly between RTHα HEPs and control HEPs (Supplemental Figure 2).
In summary, under forced differentiation conditions, RTHα cells differentiate nearly as well as control cells.

T3 enhances spontaneous differentiation of RTHα HEPs

It has been shown that addition of T3 does improve the differentiation characteristics of erythroid progenitors when grown *ex vivo*.[252] To investigate the effect of T3 on RTHα HEPs, we exposed part of the RTHα (n = 7) and control HEP cultures (n = 5), at later stages of proliferation (~ day 14), to 10 nM T3. After 2 days, this resulted in an increased proportion of differentiated HEPs when compared to HEPs cultured without T3, with a similar effect in RTHα and control samples. Cells in treated samples were smaller (Figure 4), stained less for c-Kit, and were more positive for GPA (Supplemental Figure 3).

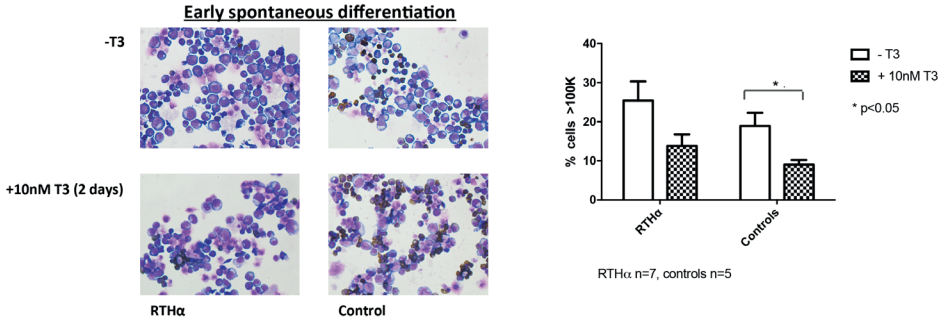


Figure 4. Cytospin preparations of RTHα-derived (P4; C392X, P5; A263S) and control HEPs at day 16 cultured with and without 10nM T3 for 2 days. Addition of T3 resulted in an increased proportion of morphologically differentiated HEPs in both RTHα patients and controls. The decrease in cell size was significant in control HEPs.

Discussion

In the current study we show that inactivating mutations in TRα affect the balance between proliferation and differentiation in human erythroid progenitor cells (HEPs). Ex vivo studies show that RTHα HEPs have a reduced capacity to differentiate spontaneously in comparison to control HEPs.
Several studies in humans have documented an association between hypothyroidism and reduced red blood cell counts, identifying mild anemia in 20–60% of hypothyroid patients.[201,258] Studies in avian and murine models point towards involvement of TRα in later stages of erythropoiesis.[203–206,259,260] However, it is important to point out that there are significant differences in the physiology of normal erythropoiesis between murine

and human contexts. For example, in mice, stress-induced erythropoiesis occurs in spleen, which is not the case in humans.[203]

Since (mild) anemia, mostly normocytic normochromic, is also a common characteristic in RTHα patients,[130,151,153,188,189,192,221,253] we decided to investigate if a defect in TRα affects the red cell maturation in RTHα patients. In this context, we sought to study whether tissue-specific hypothyroidism due to mutations in TRs affects erythropoiesis to a similar extent as primary hypothyroidism. The use of a well-validated cell culture protocol[254,255] allowed us to expand the limited number of hematopoietic progenitors present in peripheral blood samples of both RTHα patients and healthy controls.

We were able to synchronize the development of HEPs at the pro-erythroblast stage, when cells show the first morphologically recognizable features of erythroid cells. From this stage, we studied the maturation of the HEPs using serum-free medium containing proliferation factors. Remarkably, RTHα HEPs continued to proliferate for a prolonged period whereas control HEPs started to differentiate spontaneously from day 14. This delay in maturation and development was evident from both morphological appearance and expression of cell surface markers at this stage.[242,254] The serum-free culture medium used in our experiments contained low levels of T3 (~0.5nM), which may have been sufficient to enhance differentiation in control HEPs by activation of wild-type TRα. However, in RTHα-derived HEPs such low T3 concentrations were probably unable to induce differentiation to a similar extent as these cells harbored mutant TRα with reduced T3 affinity, resulting in a prolonged proliferation phase.[106]

For efficient terminal maturation, proliferation factors in the medium need to be replaced by differentiation factors (higher concentration of Epo, transferrin, serum). Addition of serum is necessary for complete down-regulation of c-Kit and a further induction of GPA in human erythroblasts, both characteristics of terminal differentiation.[255] Under such optimal differentiation conditions, differences between RTHα-derived and control HEPs decreased. This suggests that TRα mutations most likely affect the timing of onset of differentiation, and influence the actual differentiation process itself to a lesser extent.

To investigate whether higher concentrations of T3 could overcome such delayed maturation dynamics, cultured HEPs during late proliferation were exposed to 10 nM T3. After 2 days, HEPs of both RTHα-derived and control T3-treated cells showed a modest decrease in cell size indicating an increased proportion of spontaneous differentiation. This observation is consistent with previous findings from Leberbauer et al,[255] showing that proliferating HEPs treated with T3 have a gradual decrease in cell size, reduced proliferation rates and higher hemoglobin content.

Previous studies indicate that exposure of normal HEPs to T3 does not affect expression of differentiation cell surface markers[255] and we confirmed these findings with control HEPs in this study. In contrast, RTHα HEPs exposed to 10 nM T3 showed a slight

increase in CD71 and GPA expression together with down regulation of c-Kit levels. One explanation for these findings is that levels of T3 present in normal medium are sufficient to permit differentiation of control HEPs with higher exogenous T3 concentrations following addition of exogenous hormone only shifting the balance of cells achieving terminal maturation. On the other hand, in RTHα-derived HEPs, harboring dysfunctional TRα, higher T3 concentrations could help overcome the TRα defect, thereby accelerating the onset of spontaneous differentiation. Previous characterization[130,151,153,188,189,192,193,221,253] of TRα mutations identified in RTHα cases indicates variable dysfunction with frame shift and nonsense mutations being markedly more deleterious than missense mutations (with some proximal receptor mutations also affecting TRα2). In this context, both in the current study and in reports of other published RTHα cases, there is no association between the degree of anemia and the severity of mutations in TRα. Furthermore, there is no relationship between hemoglobin concentrations or RBC number and TH levels in cases, all showing inherent variation among different subjects. Given these in vivo findings in RTHα, it is not surprising that the maturation dynamics of RTHα HEPs, harboring TRα mutations of varying severity, did not differ. Despite the anemia, the number of reticulocytes was not increased in RTHα patients, suggesting that the release of immature erythrocytes into the circulation is not altered. In addition, no major abnormalities in other blood cell lineages were detected. However, we were not able to measure the life span of erythrocytes in our patients in vivo; accordingly, we cannot discount the possibility that the life span of RTHα erythrocytes is altered compared to healthy controls.

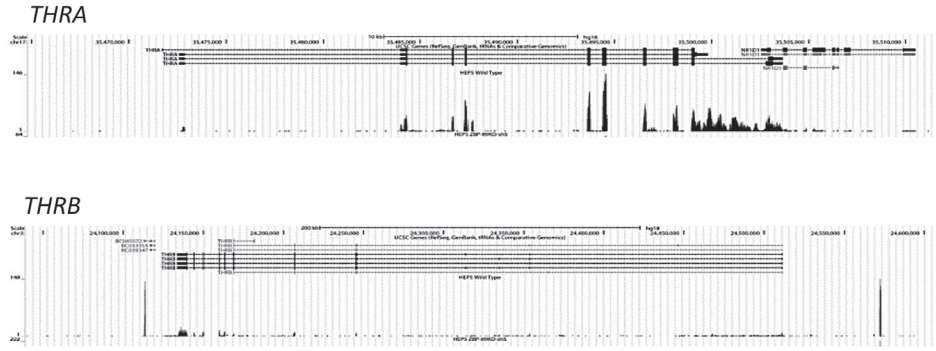
Cultured, RTHα-derived HEPs responded to 10 nM T3 exposure, whereas LT4 treatment did not correct anemia in most patients with RTHα. One possible explanation for this discrepancy is that mutant TRα affects earlier phases of erythrocyte development in the bone marrow in RTHα patients, which we have not been able to examine in this study. In the current project we studied relatively late stages of erythrocyte maturation, in which T3 is known to favor differentiation of erythrocytes.[261] In this phase, addition of higher T3 concentrations stimulated the onset of differentiation in both RTHα-derived and control HEPs. However, it remains unclear whether T3 via TRα similarly affects earlier stages of erythrocyte development and if so, whether LT4 treatment of RTHα patients can rescue mutant TRα function in this earlier phase.

In addition, we cannot discount the possibility that culture of isolated HEPs ex vivo enables different facets of the maturation process to be studied and regulated by exposure to T3, whereas it is more difficult to influence the likely complex interactions between HEPs with other cell types and factors that control the maturation process in vivo, with TH treatment. Thus, in addition to intrinsic differences between RTHα and normal erythrocyte progenitors, it is conceivable that such added interactions contribute to the anemia of RTHα patients.

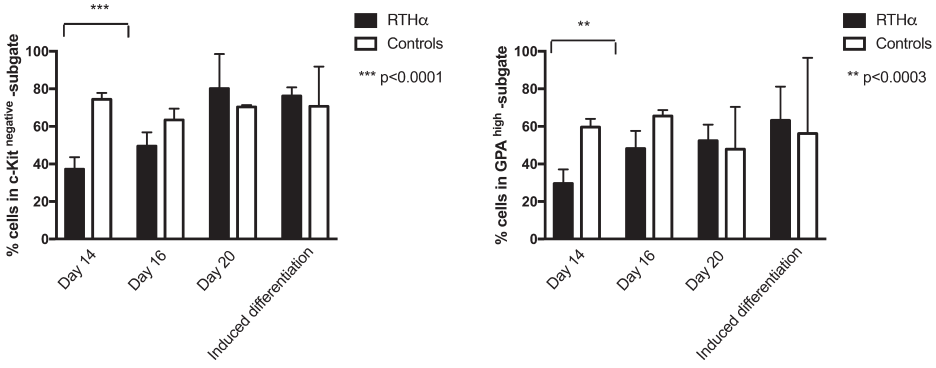
In conclusion, by studying erythroid progenitor cells from RTHα patients ex vivo, we have shown that both TH and TRα play a role in the later phases of human erythropoiesis. Erythrocyte progenitors from RTHα patients exhibit defective maturation capacity, with later onset of, and a slower progression through, the terminal differentiation program.

Acknowledgements

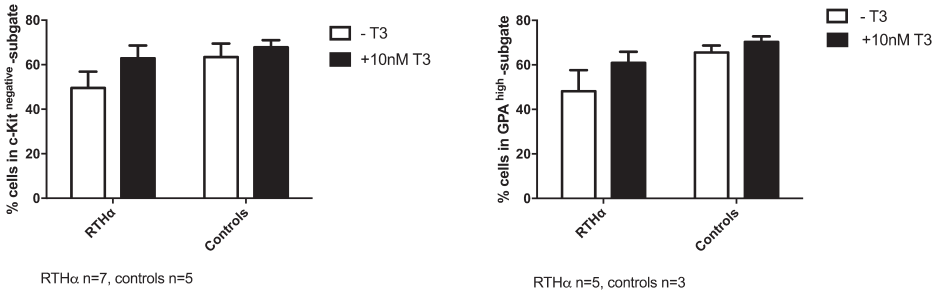
A.L.M. van Gucht, M.E. Meima, and R.P. Peeters are supported by a Zon-MWTOP grant (number 91212044) and an Erasmus MC MRACE Grant. A.L.M. van Gucht and R.P. Peeters are also supported by an ETA Research Grant. K. Chatterjee is supported by a Welcome Trust Investigator Award (095564/Z/11/Z); K. Chatterjee and C. Moran are supported by the NIHR Cambridge Biomedical Research Centre.



Supplemental Figure 1. RNA expression of THRA and THRB in HEPs from a control during late proliferation.



Supplemental Figure 2. The expression of cell surface markers during different stages of HEP maturation. During spontaneous differentiation, values for c-Kit and GPA expression in RTHα-derived HEPs reached comparable levels as control samples with a delay of 4 days. During forced differentiation, the expression of the different cell surface markers did not significantly differ between RTHα-derived and control HEPs.



Supplemental Figure 3. 10nM T3 was added to part of the RTHα-derived and control HEP cultures at day 14 (late proliferation). After 2 days, RTHα-derived HEPs cultured on T3 showed a decreased c-Kit expression and a mildly increased CD71 and GPA expression. This was less evident in (the yet more differentiated) control HEPs.

RESISTANCE TO THYROID HORMONE DUE TO HETEROZYGOUS MUTATIONS IN THYROID HORMONE RECEPTOR ALPHA

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Current Topics in Developmental Biology 2017

8

Abstract

Background

Thyroid hormone (TH) acts via nuclear thyroid hormone receptors (TRs). TR isoforms (TRα1, TRα2, TRβ1, TRβ2) are encoded by distinct genes (*THRA* and *THRB*) and show differing tissue distributions. Patients with mutations in *THRB*, exhibiting resistance within the hypothalamic-pituitary-thyroid axis with elevated TH and non-suppressed TSH levels, were first described decades ago. In 2012, the first patients with mutations in *THRA* were identified.

Scope of this review

This review describes the clinical and biochemical characteristics of patients with Resistance to Thyroid Hormone alpha (RTHα) due to heterozygous mutations in *THRA*. The genetic basis and molecular pathogenesis of the disorder together with effects of levothyroxine treatment are discussed.

Conclusions

The severity of the clinical phenotype of RTHα patients seems to be associated with the location and type of mutation in *THRA*. The most frequent abnormalities observed include anaemia, constipation and growth and developmental delay. In addition, serum (F)T3 levels can be high-normal to high, (F)T4 and rT3 levels normal to low, whilst TSH is normal or mildly raised. Despite heterogeneous consequences of mutations in *THRA*, RTHα should be suspected in subjects with even mild clinical features of hypothyroidism together with high/high-normal (F)T3, low/low-normal (F)T4, and normal TSH.

Introduction

Thyroid hormone (TH) is essential for normal development, growth and cellular metabolism. The importance of TH for the developing brain is exemplified by the severe defects in motor and mental development seen in patients with untreated congenital hypothyroidism.[1,2]

Serum TH levels are regulated by the hypothalamic-pituitary-thyroid (HPT) axis, where hypothalamic thyrotropin releasing hormone (TRH) stimulates the production of pituitary thyroid-stimulating hormone (TSH), which in turn stimulates the thyroid gland to produce TH. The thyroid predominantly synthesises the prohormone T4, with only a small fraction of T3, the bioactive hormone, being produced.[5,6] TRH and TSH synthesis is regulated by T3 and T4 as part of a negative feedback mechanism.

The intracellular availability of TH is dependent on transmembrane transport, mediated by transporters such as monocarboxylate transporter 8 (MCT8). Intracellular TH concentrations are tightly regulated by three deiodinase enzymes (DIOs), mediating the conversion of T4 to T3 (DIO1 and DIO2) and of T3 and T4 into inactive metabolites (DIO3). In the nucleus, T3 binds to T3 receptors (TRs), which belong to the nuclear receptor superfamily of ligand-inducible transcription factors. TRs preferentially form heterodimers with retinoid X receptors (RXRs) and regulate target gene expression by binding to T3 response elements (TREs), usually located in their promoter region. In the absence of TH, unliganded TRs repress basal transcription of positively regulated genes by recruiting corepressors (e.g. nuclear receptor corepressor [NCoR], silencing mediator for retinoic acid and thyroid receptors [SMRT]) and histone deacetylase (HDAC). Binding of T3 to TRs results in dissociation of the corepressor complex and recruitment of coactivator proteins (e.g. steroid receptor coactivator 1 [SRC-1], and the histone acetyl transferase [HAT], CREB-binding protein [CBP] and CBP-associated factor [pCAF]), which induce transcriptional activation.[7,105–107] TH also exerts non-genomic effects,[262,263] but these are beyond the scope of this review.

In humans, TRs are encoded by two genes (*THRA* and *THRB*) on chromosomes 17 and 3, respectively. Two receptor isoforms are generated from the *THRA* locus by alternative splicing: TRα1 is predominantly expressed in the central nervous system (CNS), bone, heart, skeletal muscle and gastrointestinal tract, whereas the non-T3 binding isoform TRα2 is expressed in various tissues (e.g. brain and testis). Two different isoforms TRβ1 and TRβ2, which diverge in their amino-terminal regions, are generated from the *THRB* locus, TRβ1 is considered the major isoform in liver, kidney and thyroid. TRβ1 is also predominantly expressed in the brain, pituitary and inner ear, where it mediates overlapping functions with TRβ2.[264] TRβ2 has a more restricted expression pattern regulating neurosensory (hearing, vision) development as well as the HPT-axis.[67,108,109]

Since 1986 it has been known that heterozygous mutations within three, CpG-rich, hotspots of the ligand-binding domain (LBD) of TRβ result in resistance to thyroid hormone β (RTHβ), a clinical syndrome of refractoriness to TH that was recognized two decades earlier.[265] The incidence of RTHβ is ~ 1 in 40,000 and several hundred mutations have been identified so far.[7,266] Patients with RTHβ are characterized by elevated serum TH levels with non-suppressed TSH, and a phenotype ranging from being asymptomatic to having clinical features of thyrotoxicosis (e.g. failure to thrive in infancy; tachycardia and cardiac arrhythmia, raised metabolic rate, anxiety in adulthood).[7,110]

Human TRα and TRβ are highly homologous, with the proteins showing 80% amino acid identity. Taking into account the large number of different TRβ mutations associated with RTHβ, the identification of analogous mutations in human TRα had been anticipated. In an attempt to predict the clinical consequences of defective TRα, several knock-in and knock-out mouse models have been generated. Mice harboring different, heterozygous TRα mutations exhibit diverse phenotypes, depending on the severity and location of the mutations; however, all have near-normal thyroid function tests.[111–114] Perhaps due to this lack of a clear biochemical thyroid phenotype, no mutation in human *THRA* was reported until 2012.

In the last 4 years, 28 cases from 15 different families with mutations in *THRA* have been identified. In this review, we present an overview of the clinical features together with the underlying molecular mechanisms and effects of treatment in patients with Resistance to TH due to heterozygous mutations in TRα (RTHα).

Molecular mechanisms underlying RTHα

Table 1 shows all mutations in *THRA* identified in patients so far. Affected individuals are heterozygous for the mutations, which occurred *de novo* in 8 cases or were familial in 7 cases. All mutations identified so far are localized in the ligand-binding domain (LBD) of *THRA*, as illustrated in Figure 1. Consistent with this, T3 binding by the mutant receptors is impaired whereas DNA binding is unaffected.

The mutations can be categorized into 3 classes of receptor defects: 1) frame shift / premature stop mutations, in which a deletion or insertion of one or several nucleotides results in a truncated receptor, 2) nonsense mutations, in which a nucleotide substitution results in a premature stop codon, and 3) missense mutations, in which a nucleotide substitution leads to an amino acid change without affecting protein length.

All patients are heterozygous, suggesting a dominant-negative effect of the mutant receptors on wild-type TRα. Indeed, when co-expressed the mutant receptors inhibit the function of their wild-type counterparts in a dominant-negative manner,[130,151,153,154,188,189,192,193,221] similar to what has been reported for TRβ mutations in

RTHβ. T3-induced dissociation of mutant TRα receptors from corepressors is retarded and ligand-dependent recruitment of coactivators impaired, likely contributing to the dominant-negative inhibition. *Ex vivo* analysis of mutation-containing, patient-derived,

Table 1. Mutations in *THRA* identified in patients. Mutations are organized by the type of mutation: frame shift, nonsense and missense.

Missense	Nonsense	Frame shift
G207E (unpublished) ♂ 15 mo + mother (26 yrs)	C392X ♂ 18 yrs	C380fs387X ♀ 12 yrs
D211G[221] ♀ 18 mo + father (30 yrs)	E403X ♀ 6 yrs	A382fs388X ♀ 45 yrs
A263V[192] ♀ 60 yrs + 2 sons (26/30 yrs)	E403X ♀ 15 yrs	F397fs406X,153 ♀ 11 yrs + father (42 yrs)
A263S[154] ♂ 2.6 yrs + sister (7.4 yrs), mother (31 yrs), grandfather (55 yrs), aunt (35 yrs), 2 cousins (17 / 8.8 yrs)		
N359Y[193] ♀ 27 yrs		
R384C[284]		
R384H[154] ♂ 15 mo + mother (30 yrs)		
E403K[189] ♀ 6 yrs + father (39 yrs)		
P398R[189] ♀ 5 yrs		

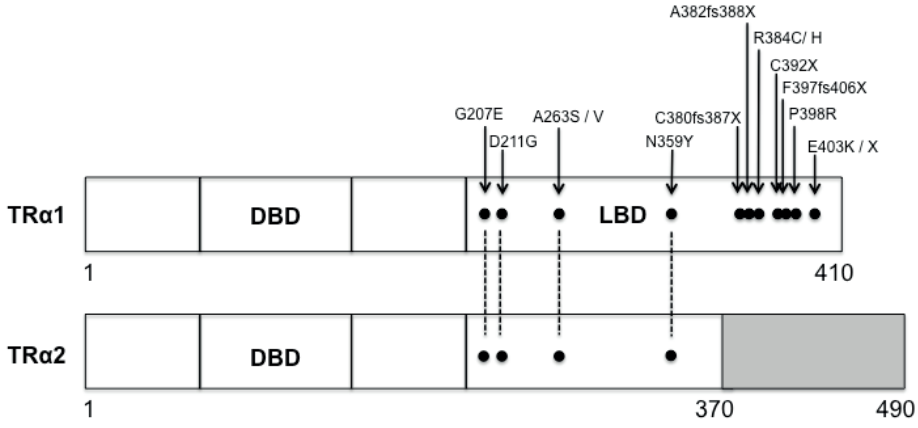

Figure 1. Localization of identified mutations in *THRA*. Thyroid hormone receptor α1 (TRα1), the T3 receptor encoded by the *THRA* gene and the splice variant TRα2, which does not bind T3 and does not act as a receptor, have identical aminoterminal and DNA binding domains. The non-homologous part of the ligand binding domains (LBD) is coloured in grey. The location of all known TRα mutations is indicated. G207E, D211G, A263S, A263V, and N359Y affect both TRα1 and α2 transcripts.

cells showed reduced T3-mediated induction of target gene expression, consistent with dominant negative inhibition occurring *in vivo*.[151,188,192]

In 5 families, affected individuals harbored missense mutations, localized further upstream in *THRA,* thereby involving both TRα1 and TRα2 proteins. At low T3 concentrations such missense mutant receptors showed reduced transcriptional activity together with moderate dominant-negative inhibition of wild-type TRα1. Interestingly, at higher T3 concentrations, mutant receptors exhibited transcriptional activity similar to wild-type TRα1 with reversal of their dominant negative inhibitory activity.[154,192,221] These observations suggest that patients harbouring such milder, missense mutations may particularly benefit from TH treatment. Similar observations were made in mice with a missense mutation (R384C) in TRα1, with locomotor defects and behavioral abnormalities being alleviated by TH treatment.[111,207,208]

The phenotype of patients with a mutation in both TRα1/2 resembles the clinical characteristics of RTHα patients with a mutation in TRα1 alone.[154,192,221] One exception to this is a patient with a mutation (N359Y) in TRα1/2 who showed a distinct clinical phenotype comprising skeletal malformations (clavicular agenesis, metacarpal fusion, syndactyly of digits), chronic diarrhoea, macrocytic anaemia, and hypercalcaemia.[193] It remains uncertain whether these additional phenotypic characteristics are due solely to the mutation in *THRA*. In all other RTHα cases described to date, the mutation in TRα2 does not seem to contribute to the phenotype, which is concordant with observations in TRα2KO mice. In these mice, phenotypic abnormalities are most probably not due to loss of TRα2, but are a consequence of compensatory overexpression of TRα1.[209] In addition, previous studies have suggested that TRα2 is unable to heterodimerise with RXR, bind TREs or exert dominant-negative activity via corepressor recruitment.[237,238,267] Noteworthy, the severity of the clinical RTHα phenotype appears to be associated with the type and localization of the mutation in TRα. In this, RTHα patients with frameshift and nonsense mutations show a more affected phenotype in comparison to RTHα patients with milder missense mutations.

Clinical phenotype

Appearance

Most patients were born at term after an eventless pregnancy and displayed no abnormal characteristics, although recognized features of hypothyroidism (e.g. macroglossia, poor feeding, hoarse cry) were present in some cases at birth (Moran et al 2013; Moran et al 2014). Physical characteristics associated with hypothyroidism (e.g. macrocephaly, hypertelorism, a broad face and flattened nasal bridge) became more evident with time, together with poor linear growth, resulting in a dysmorphic appearance (see

Table 2. Clinical and biochemical characteristics of patients with resistance to thyroid hormone alpha.

System	Clinical / biochemical characteristics
Appearance: Dysmorphic syndrome	• Flat nasal bridge • Broad face, thickened lips • Macroglossia • Coarse facies • Skin tags
Neurological and cognitive	• Delayed developmental milestones • Delayed speech development, dysarthric speech • Slow initiation of movement • Fine and gross motor dyspraxia, broad-based ataxic gait • Dysdiadochokinesis • Reduced global IQ, reduced verbal and performance scores
Skeletal	• Disproportionate short stature: reduced total height, normal sitting height, reduced subischial leg length. • Macrocephaly: delayed fontanelle fusion, thickened calvarium, wormian bones • Delayed tooth eruption • Delayed ossification: delayed bone age, femoral epiphyseal dysgenesis • Cortical hyperostosis in long bones, increased bone mineral density
Cardiac	• Bradycardia • Low/low-normal blood pressure
Gastrointestinal	• Constipation: decreased peristalsis, delayed intestinal transit, bowel dilatation
Biochemical and metabolic	• Thyroid function tests: normal or slightly raised TSH, low/low-normal (F)T4, high/high-normal (F)T3, low/low-normal rT3, decreased (F)T4/(F)T3 ratio and increased (F)T3/rT3 ratio • Hematological: low red cell mass or hematocrit with normal/raised MCV, B12, folate and reticulocyte count • SHBG: high/normal • IGF-1: low/normal • Low metabolic rate

Table 2).[130,151,153,154,188,192,193,221] Several patients have numerous skin tags and moles, which seem to increase in number with age.[151,188,192]

Neurological and cognitive

In almost all index patients a delay in reaching developmental milestones was the prime reason for consultation. Childhood cases show a slow initiation of motor movement together with impaired fine and gross motor coordination, resulting in dyspraxia and a broad-based ataxic gait. One young girl with a missense (D211G) mutation[221] initially presented with severe, predominantly axial, hypotonia. Two cases displayed childhood seizures.[188,221] Several patients show mild to moderate/severe intellectual disability with a lower global IQ and reduced verbal and performance scores. A delay in speech development and dysarthric speech are consistent features in the majority of patients (see Table 2).[130,151,153,154,188,189,192,221]

Skeletal

Most, but not all, RTHα patients have short stature; some have a disproportionate reduction in subischial leg length.

Skull radiographs of RTHα patients in childhood show delayed fontanelle fusion together with excessively serpiginious skull sutures (wormian bone appearance). Retarded ossification, resulting in delayed bone age or femoral epiphyseal dysgenesis, can be present. In addition, a thickened calvarium and a delayed tooth eruption have been observed in most cases.[130,151,153,188,189,192,221] Especially in adults, cortical hyperostosis in long bones together with increased bone mineral density has been documented (see Table 2).

Cardiac and gastrointestinal

Blood pressure and resting heart rate are normal or low/low-normal in most RTHα patients, which is remarkable in view of the high serum T3 levels (see below).

In the majority of both childhood and adult patients, constipation is a persistent problem due to decreased frequency of bowel movements.[130,151,153,154,188,189,192,221] When evaluated in patients, abdominal radiography shows a delayed intestinal transit together with bowel dilatation. In addition, reduced peristalsis is seen on colonic manometry (see Table 2).[151,188] Only one adult case has chronic diarrhoea.[193]

Biochemical and metabolic

Thyroid function tests show a consistent pattern, albeit with significant variation between different cases. The thyroid biochemical phenotype not only varies between patients with different mutations but also between patients carrying the same mutation. TSH levels are in the normal range in all patients, except for one adult female patient[188] who showed a slightly raised TSH while off LT4 treatment. In almost all index cases, (F)T4 levels are initially low or low-normal. A rise in (F)T4 levels into the normal range is observed in some of the cases during follow-up, possibly indicating attenuation of the biochemical phenotype with time. (F)T3 levels are high or high-normal in most patients, although normal (F)T3 levels have also been reported in several cases.[154] Nevertheless, an abnormally high T3/T4 ratio seems a consistent finding among all patients. Reverse T3 (rT3) levels are normal or subnormal, resulting in a higher serum T3/rT3 ratio (see Table 2).[151,188,189,192,221]

Other markers, reflecting TH action in peripheral tissues, can also be altered. A mild, usually normocytic normochromic, anaemia is an almost universal characteristic in RTHα patients. Serum muscle creatine kinase levels and sex hormone binding globulin (SHBG) levels may be raised, while circulating IGF-1 levels can be decreased.[130,151,153,154,188,189,192,193,221] Total and LDL cholesterol levels may be increased, in both childhood and adult cases.[130,153] In several patients, a low basal metabolic rate (BMR),

measured by indirect calorimetry, was recorded (see Table 2).[151,188,192,193] Overall, both clinical and biochemical abnormalities seen in RTHα are consonant with thyroid hormone resistance in tissues expressing predominantly TRα1 (e.g. heart, bone, muscle, intestine, brain), while TRβ-expressing tissues (e.g. hypothalamus, pituitary, liver) remain sensitive to TH (Figure 2).

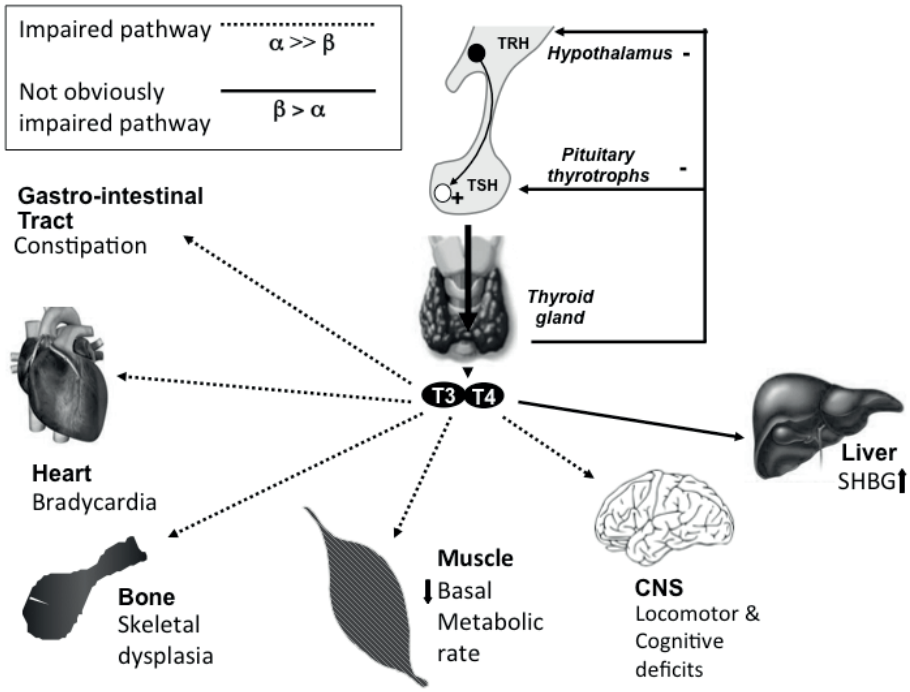

Figure 2. Cartoon showing the major tissue actions of thyroid hormone together with the predominant receptor subtypes mediating these effects. In RTHα, tissues expressing mainly TRα are resistant to thyroid hormone action ("impaired") while TRβ-expressing tissues are sensitive ("not obviously impaired").

Pathogenesis

Many clinical features in RTHα patients, such as delayed motor development, delayed cranial suture closure resulting in macrocephaly, delayed tooth eruption, developmental skeletal abnormalities (e.g. femoral epiphyseal dysgenesis, wormian bones), delayed bone age, and lower segment growth retardation, are characteristic of untreated hypothyroidism.[268,269] In addition, constipation due to decreased colonic motility and colonic dilatation is also clearly associated with hypothyroidism.[270]

Other characteristics of hypothyroidism, including slow speech, bradykinesia, dry skin and slow relaxing reflexes are also reported in RTHα cases. Interestingly, many of these

features (delayed growth, tooth eruption, fontanelle closure and endochondral and intramembranous ossification) are also present in different mouse models with frame-shift mutations (TRα1-PV) or missense mutations in TRα1 (TRα1-R384C, TRα1-P398H, TRα1-L400R).[111–114,212,271–274] Abnormal motor development in patients resembles the psychomotor phenotype of TRα1-R384C mice, which show reduced grip strength, poor limb coordination, and an abnormal gait.[111,207]

Several patients exhibit delayed mental development with cognitive deficits and two patients suffer from seizures, which have also been described in TRα1-R384C mice.[207,208] Constipation and bowel dilatation with reduced peristalsis that has been described in RTHα patients is very similar to the intestinal phenotype of $TR\alpha^{-/-}$ mice.[273,275]

Despite the variation between patients, the biochemical abnormalities (increased T3/T4 and T3/rT3 ratios) found in most RTHα cases may reflect altered peripheral TH metabolism by deiodinases. As indicated by marked suppression of serum TSH by relatively small increases in serum (F)T4 in cases, central feedback regulation of the HPT-axis is preserved, favouring a minor contribution of TRα1 but predominant role for TRβ isoforms in regulation of the HPT-axis.[153,200,264] Increased T3 and decreased rT3 levels could be due to up-regulation of hepatic DIO1 or reduced DIO3 activity as has been shown in studies with TRα mutant mice.[112,276] DIO1 is expressed in liver, kidney, and thyroid and plays an important role in the production of the bioactive T3 from T4 and the clearance of rT3. DIO3 is present in brain, skin, placenta, and pregnant uterus where it inactivates T3 and T4 and hereby protects these tissues from excess active TH. T3/T4 and T3/rT3 ratios are considered to be sensitive indicators of peripheral TH metabolism and are relatively independent of variations in serum binding proteins or thyroidal T4 production.

The abnormalities in thyroid function seen in RTHα patients do not completely correlate with observations made in different TRα mutant mouse models, since overt thyroid dysfunction was only documented in TRα1-PV and TRα1-P398H mice and not in TRα1-R384C and TRα1-L400R animals.[111–114,207] It has been shown that TRα1-PV mutant mice have markedly increased levels of kidney and liver DIO1, while T3-induced DIO3 expression in cortex is decreased, resulting in a decreased clearance of T3.[277]

Anaemia, with normal haematinics (iron, vitamin B12, folate) and haemolytic indices (reticulocyte count, circulating haptoglobin and lactate dehydrogenase), is a near-universal characteristic in RTHα. Usually a normocytic normochromic anaemia, although the mean corpuscular volume was raised in 3 cases.[151,188,193] Several human studies have documented an association between hypothyroidism and anaemia.[201,202] In addition, data from animal models support an important role for TRα in erythropoiesis.[203–206] The molecular mechanisms underlying anaemia in RTHα patients remain to be elucidated.

Spontaneous conception and uneventful pregnancy has been observed in untreated females with RTHα, despite varying features of hypothyroidism.[154] Maternal and paternal

inheritance of TRα mutations suggests that transmission of TRα mutations from parents to offspring is less impaired in humans than in mice.[112]
The observation that several affected relatives of RTHα index cases reached adulthood without identification of medical problems supports the mild character of these mutations.[154,221] Similar to RTHβ, individuals harbouring the same mutation can exhibit significant variation in severity of clinical phenotypes and it is conceivable that additional variation (e.g. in genes encoding cofactors of TH action) could mediate this.[7]

Treatment

Several RTHα patients have been treated with LT4 and as yet, no other therapeutic approaches have been explored.[151,153,154,188,192,193,221] Overall, 11 patients have been LT4-treated: in 8 cases LT4 treatment was initiated from (early) childhood and in 3 cases LT4 was started in adult life. The duration of LT4 treatment in childhood cases ranges from 1 year in a girl with a D211G mutation to 5 and 6 years in girls with F397fs406X and E403X mutations in TRα, respectively, and 12 years in a girl with a C380fs388X mutation.[130,151,154,221] Adult cases have received LT4 supplementation for 1 month to over 40 years.[154,188,192,193] LT4 treatment had beneficial effects on several RTHα characteristics, as described below. In addition, initiation of LT4 therapy at a young age also had beneficial developmental effects in childhood cases.
LT4 treatment, even in typical physiological doses, resulted in suppressed TSH levels in all RTHα patients, implying an intact feedback mechanism within the HPT axis.[151,154,188,192,193,221] In addition, serum (F)T4 and rT3 levels normalize while serum T3 remains elevated. Some peripheral markers of TH action also responded to LT4 therapy, with a rise in serum SHBG and IGF1 levels together with a decrease in serum creatine kinase and LDL cholesterol levels.[151,153,154,188,192,221] In most cases, LT4 therapy has a beneficial effect on constipation but has no effect on anaemia and a blunted effect on cardiac function.
LT4 treatment initiated at young age resulted in a catch-up growth and improved overall height in several patients, and the addition of growth hormone did not further improve growth.[151,153,221] Moreover, in one childhood case, harboring a TRα mutation whose dysfunction is reversible at higher TH levels, during LT4 treatment muscle hypotonia improved and motor development accelerated.[221] LT4 treatment from early childhood in 3 related patients, with a relatively mild mutation (A263V) in TRα, may have ameliorated their phenotype and alleviated some symptoms (e.g. dyspraxia) in adulthood.[192]
In contrast, initiation of LT4 at the age of 2.5 years in a girl with the most upstream C380fs387X frameshift mutation did not ameliorate her growth retardation, and car-

diac and renal problems, which may reflect ongoing tissue hypothyroidism, despite LT4 treatment. Concordant with this LT4 treatment of TRα1-PV mutant mice, harboring a deleterious frame-shift mutation, did not improve skeletal abnormalities or other abnormal phenotypes.[212,278,279] In patients with frame-shift mutations involving the carboxyterminal region of TRα1, the severity of the clinical phenotype and also the effect of LT4 therapy may vary depending on the exact location of these mutations and the extent to which this domain is disrupted.[153,154,188]

Several affected relatives of RTHα index patients with missense mutations, who had only been diagnosed after screening of family members of the index case, showed an amelioration of clinical features with time. Similar observations have been made in TRα1-R384C mice, which showed severe but transient impairment of post-natal development and growth. Alterations in TH levels were also only evident in these mice during the juvenile period.[111,207,208] The mechanisms underlying such age-dependent amelioration of phenotypes associated with some TRα1 mutations are unknown.

Overall, the effects observed with LT4 treatment of RTHα patients corresponds with the expected pattern, with TH resistance in organs predominantly expressing TRα1 (bone, skeletal muscle, gastrointestinal tract, myocardium, brain) and retention of TH sensitivity in TRβ-expressing tissues (hypothalamus, pituitary, liver). Indeed, chronic exposure to excess TH in LT4-treated RTHα patients could result in unwanted toxicities in TRβ-expressing tissues. The observation that LT4 treatment in RTHα further raises SHBG and (F)T3 levels with suppression of TSH, supports this possibility.

In this regard, future therapies that could selectively target TRα1, e.g. high affinity TRα1-selective agonists, are of great interest. Such TRα1-specific thyromimetics could activate normal TRα1 and potentially, in higher dosages, overcome the impaired function of mutant TRα1. Alternative therapeutic strategies could involve targeting the dominant-negative effect of mutant TRα1 on its wild-type counterpart, either by inhibiting histone deacetylase activity or abrogating the interaction of mutant TRα1 with the corepressor complex. Studies in mice harbouring the TRα1-PV mutation showed that aberrant recruitment of NCoR by mutant TRα1 contributes to the phenotype and the severity of this can be partially reversed by introducing a mutation in NCoR that abrogates its interaction with TRα1.[280] In an alternative approach, TRα1PV-mediated repression could be relieved by administration of suberoylanilide hydroxyamic acid (SAHA), a histone deacetylase inhibitor, also ameliorating the phenotypic abnormalities in these mice.[281–283]

Conclusions

The clinical consequences of mutations in *THRA* are variable in extent and severity. This applies both to patients with different TRα mutations and to individuals in a family harbouring the same mutation. Similar to RTHβ, the observation that affected relatives with a relatively mild phenotype in some RTHα families were only diagnosed by genetic screening after initial diagnosis of the index cases supports the hypothesis that the incidence of RTHα could be much higher than currently estimated. Given the strong homology between the TRα1 and TRβ receptors and similar localization pattern of both TRα and TRβ mutations, the incidence of RTHα could correspond to that of RTHβ (1:40,000).

RTHα should be suspected in subjects when even mild clinical features of hypothyroidism are present along with a normal TSH, a high serum T3/T4 or T3/rT3 ratio. RTHα patients, particularly those with mild mutations or diagnosed in early life, may benefit particularly from LT4 treatment. Further research to develop alternative treatment options and clinical biomarkers which better reflect the effects of LT4 treatment in both TRα- and TRβ-expressing tissues will be of great value.

Acknowledgements

A.L.M. van Gucht, M.E. Meima, and R.P. Peeters are supported by a Zon-MWTOP Grant (number 91212044) and an Erasmus MC MRACE Grant. C. Moran and K. Chatterjee are supported by the NIHR Cambridge Biomedical Research Centre.

GENERAL DISCUSSION

9

Introduction

Thyroid hormone (TH) is essential for normal development and function of all tissues in the human body. The intracellular TH availability is dependent on transmembrane transport proteins and deiodinating enzymes (DIOs) that mediate the conversion of T4 to T3 (DIO1 and DIO2) and the degradation of T3 and T4 into inactive metabolites (DIO3). TH action takes place in the nucleus, where the bioactive T3 binds to thyroid hormone receptors (TRs) and thereby modulates transcription of TH target genes. This thesis mainly focusses on two key players in TH homeostasis: TH transport and TH action, in both health and disease.

First, we investigated whether the well-known TH transporters MCT8 and MCT10 increase the availability of TH for the nuclear TRs rather than generate a rapid equilibrium between TH in- and efflux. Furthermore, we studied several liver-specific TH transporters in their transport of both TH and TH analogs, such as eprotirome and GC-1.

Next, we identified a number of patients with mutations in the thyroid hormone receptor alpha (TRα). We investigated the impact of the different mutations on TH action and made phenotype-genotype correlations. Finally, we studied the role of TH and TRα in human erythropoiesis.

In this chapter, the relevance of the performed studies is discussed in view of the current literature and further research strategies are explored.

Thyroid hormone transport

Lack of thyroid hormone (TH) in early life results in severe developmental impairment.[1,115,116] To mediate its effects, TH must first be transported across the plasma membrane by TH transporters. Several TH transporters from different families have been identified, such as monocarboxylate transporters (MCT), L-type amino acid transporters (LAT), organic anion transporting polypeptides (OATP), and bile acid transporters (such as the sodium taurocholate co-transporting polypeptide, NTCP)..[117]

MCT8 and MCT10

MCT8 and MCT10 are two highly homologous monocarboxylate transporters. Both transporters show high activity and specificity for TH transport, while MCT10 also transports aromatic amino acids. MCT8 is important for TH supply to the brain, since this transporter is expressed in endothelial cells of the blood-brain barrier (BBB) as well as in neurons.[14,24,25,118]

Apart from influx, MCT8 and MCT10 also facilitate efflux of TH.[24,36] Therefore, it remained unclear whether MCT8 and MCT10 increase the availability of TH at the nuclear level

or merely establish a more rapid equilibrium between uptake and efflux at the plasma membrane. In **chapter 2** we addressed this question, using cells transfected with MCT8 or MCT10, together with TRβ1 and a T3 response element-driven luciferase construct to measure T3-dependent transcriptional activity. In addition, to study the effects of MCT8 and MCT10 on the metabolism of T3, cells were co-transfected with the type 3 deiodinase (D3), which is a plasma membrane resident protein.[127]

Our results show that overexpression of MCT8 or MCT10 markedly stimulates T3 uptake, leading to an increased intracellular T3 concentration. However, the T3 availability for the nuclear TRs was only modestly increased, indicating a rapidly established equilibrium between uptake and efflux of T3 at the plasma membrane. This was further supported by the fact that inhibition of T3 efflux by the intracellular binding protein CRYM[137] resulted in an increased availability of T3 in the nucleus. It is likely that MCT8 and MCT10 have a much greater effect on intracellular T3 availability at the cell periphery than at the cell nucleus. This is supported by the observation that intracellular T3 metabolism by D3 was greatly stimulated in cells co-transfected with MCT8 or MCT10, suggesting that the available T3 concentration at the D3 active center was much less affected by the rapid T3 efflux mediated by MCT8 or MCT10.

Mutations in MCT8 are associated with the Allan-Herndon-Dudley syndrome (AHDS), an X-linked disorder leading to severe psychomotor retardation due to a hypothyroid state in the brain during development.[30,31] Interestingly, it has been demonstrated that MCT8 mutations may differentially affect cellular uptake and efflux of T3, leading to distinct neurological abnormalities in AHDS patients.[285] In addition, studies in MCT8 knock-out mice, which have similar abnormal thyroid function tests as AHDS patients, show that T3 efflux is not affected in these animals.[26,39] These observations underscore the biological relevance of the efflux function of MCT8, which likely depends on the cellular context and may have different consequences in different tissues.[286]

MCT8 and MCT10 stimulate T3 metabolism by co-transfected D3, however, the rapid degradation by D3 also reduces the nuclear availability of T3. D3 is highly expressed in fetal tissues and the expression levels rapidly decrease after birth,[133,134,176] suggesting that prevention of premature T3 action is important for developmental processes. This is supported by the observation that D3 KO mice show phenotypic abnormalities due to excessive T3 levels during fetal and early neonatal life.[136,287] In addition, a more recent study showed that MCT8 deletion markedly improves the phenotype of D3 deficient mice, eliminating neonatal lethality and significantly ameliorating their growth retardation. Altogether, this provides more evidence for the interdependent relationship between TH transport into cells by MCT8 and the deiodination process by D3.[288]

In humans, the strongest MCT10 expression was reported in pancreas and skeletal muscle, suggesting the need for TH and/or aromatic acids transport in this organ.[29] Human MCT10 prefers T3 over T4 uptake and appears to facilitate efflux less effectively

than MCT8.[25] Interestingly, chicken MCT10 was a better T3 exporter than both chicken MCT8 and human MCT10.[289] More studies into mechanisms of TH uptake and efflux regulation by MCT10 are required for a better understanding of its role in developmental processes and the possible consequences of mutations in this transporter.

Liver specific transporters

OATPs represent a large family of homologous proteins, many of which have been shown to transport iodothyronines and their sulfate conjugates.[55,61,290,291] OATP1B1 and OATP1B3 are expressed specifically in the liver, while OATP1A2 is expressed in brain, liver and kidney. OATPs transport their substrates in a sodium-independent manner. This is in contrast to the bile acid transporter NTCP, which is also expressed in liver and facilitates TH uptake in a sodium-dependent manner.[20,22,34,77]

Besides their role in the enterohepatic circulation of bile salts, liver-specific transporters, such as OATPs and NTCP, are important in the maintenance of lipid and energy homeostasis by TH.[105,215,218] Dyslipidemia and metabolic syndrome are major health concerns and some aspects of these diseases can be ameliorated by manipulating TH signaling pathways.[170,292,293] TH plays an important role in lipid metabolism, effects of which are mediated by stimulation of the TRβ1 receptor in liver by T3. In liver, TRβ1 has direct effects on bile acid and fatty acid synthesis by regulating the expression of important factors such as CYP7a1, acetyl CoA carboxylase, fatty acid synthase, and carbohydrate response element binding protein.[215]

Therefore, liver-specific TRβ-selective analogs can be ideal candidates to reduce serum cholesterol levels without adverse thyromimetic effects.[145] To achieve this, it is necessary to target lipid-lowering TH analogs to the liver through liver-specific transporters.

In **chapter 3 and 4** we gained more insight in the characteristics of different TH transporters and their ability to transport the TH analogs eprotirome and GC-1. Eprotirome and GC-1 are thyromimetics that preferentially bind to TRβ and accumulate in the liver.[170,172,294,295] In animals, both TH analogs show a reduction of LDL cholesterol, triglycerides and lipoprotein-a.[296]

Using transiently transfected COS1, JEG3 and HEK293 cells, we showed that eprotirome and GC-1 are effectively transported into the cells by the liver-specific transporters NTCP, OATP1B1, OATP1B3, but not by OATP1A2. In addition, these transporters induced TR-mediated action of both eprotirome and GC-1, indicating that cellular uptake of eprotirome and GC-1 by these transporters resulted in an increased nuclear availability of these TH analogs. However, when using JEG3 and HEK293 cells, a significant uptake of eprotirome and GC-1 was also seen in cells transfected with empty vector. This suggests that other endogenous transporters can transport these TH analogs into other tissues, since JEG3 and HEK293 cells represent placenta and kidney cells, respectively.

Remarkably, an earlier study with eprotirome in dogs was discontinued due to unwanted side effects of eprotirome in cartilage (Karo Bio. Karo Bio terminates the eprotirome program. [Press release]: www.karobio.com; 2012). One possible explanation for this could be that other transporters are expressed in cartilage, which are also involved in eprotirome (and possibly GC-1) uptake. The expression of the liver-specific transporters NTCP, OATP1B1, and OATP1B3 is not reported in human cartilage,[163] which might indeed point towards the involvement of other transporters in this tissue. However, it cannot be excluded that the expression of NTCP, OATP1B1 and OATP1B3 is different in dog cartilage compared to human cartilage.
In humans, short eprotirome treatment of patients with hypercholesterolemia showed promising results. However, it is possible that deleterious effects can emerge during longer treatment duration.[146,162,297,298] Therefore, a better understanding of the adverse effects of eprotirome in the phase III trial in dogs must be obtained before more studies with lipid-lowering TH analogs in humans can be conducted. Was the cartilage damage a consequence of direct interactions between eprotirome and TRs or other proteins? Did eprotirome act on the HPT-axis and influenced cartilage indirectly by inducing mild hypothyroidism? Or did eprotirome have effects on cartilage via effects in the liver?
Nevertheless, our findings illustrate that TH analogs may be targeted to specific tissues, depending on the transporters they express. Still, it is important to gain further insight in the mechanisms of action of these transporters and their tissue specificity. In addition, it is necessary to investigate possible interactions between uptake of eprotirome, GC-1 and other substrates transported by these transporters, e.g. bile acids.

Thyroid hormone action

T3 has its action via nuclear TH receptors (TRs). TRs preferentially form heterodimers with retinoid X receptors (RXRs) and regulate target gene expression by binding to TH response elements (TREs) in target gene bodies.[7,105]
In humans, TR isoforms (TRα1, TRα2, TRβ1, TRβ2) are encoded by two genes (*THRA* and *THRB*) on chromosomes 17 and 3, respectively, and show differing tissue expression patterns.[67,108,109]

RTHβ

Since 1986 it has been known that heterozygous mutations in *THRB* result in Resistance to Thyroid Hormone β (RTHβ). Patients with RTHβ are characterized by elevated serum TH levels, a non-suppressed TSH, and a variable phenotype comprising goiter, tachycardia and raised energy expenditure.[7,110]

RTHα

Human TRα and TRβ are highly homologous, showing an overall 80% amino acid identity. In an attempt to predict the clinical consequences of mutations in *THRA*, several knock-in and knock-out mouse models have been generated. However all mice lacked a clear biochemical thyroid phenotype.[111–114] As a consequence, the first patients with mutations in *THRA* were only identified in 2012.

In **chapters 5 and 6** we characterized 12 newly identified RTHα patients from 4 different families with 4 distinct *THRA* mutations.[154,179] These mutations varied regarding severity, including missense mutations that led to a relatively mild phenotype in several RTHα patients. This enabled us to recognize the most frequent abnormalities, associated with *THRA* mutations, and thus allow easier identification of new patients. The characterization of a large family harboring the same mutation provided us with more insight into the variation of the RTHα phenotype. In addition, we studied the effects of levothyroxine treatment in patients with distinct mutations with different responses to therapy, depending on the severity of the mutation.

The severity of the clinical phenotype of RTHα patients seems to be associated with the location and type of mutation in *THRA*. The most frequent abnormalities observed include an abnormal combination of thyroid function tests, growth and developmental delay, constipation, and anaemia.

Several studies in humans and animal models have indicated an association between hypothyroidism and anaemia as well as an important role for TRα in erythropoiesis.[201–206] Given the observations that RTHα patients have anaemia and that both T3 and TRα are important in the development of red blood cells, we hypothesized that mutations in TRα affect the fate decision between proliferation and differentiation of erythroblasts in human erythropoiesis. To investigate this, we studied the maturation of erythroid progenitor cells (HEPs) of RTHα patients with different TRα mutations *ex vivo*. In **chapter 7** we showed that both TH and TRα are involved in human erythropoiesis. Our results point towards a delay in developmental dynamics of RTHα HEPs, displaying sustained proliferation and delayed onset of spontaneous differentiation compared to control HEPs. This delay in development dynamics of RTHα HEPs was evident from both morphological appearance and the expression of stage-specific cell surface markers. It is likely that the intrinsic difference between RTHα and control HEPs that we found, is only one of many molecular mechanisms underlying anaemia in RTHα patients.

Lastly, we present an overview of all RTHα patients identified so far (28 cases from 15 different families) in **chapter 8**.[151,153,154,178,179,188,189,192,193]

In this chapter we discuss the heterogeneous clinical consequences of mutations in *THRA*. This applies to both patients with different TRα mutations and family members with the same mutation. The observation that, similar as for RTHβ, several relatives with a relatively mild phenotype were only diagnosed because of the initial diagnosis in

the index cases supports the hypothesis that the incidence of RTHα could be much higher than currently estimated. Given the strong homology between the TRα1 and TRβ1 receptors and a similar localization pattern of both TRα and TRβ mutations, the RTHα incidence could correspond to the incidence of RTHβ (1:40,000).[7]

RTHα should be suspected in subjects when even mild clinical features of hypothyroidism are present along with a normal TSH, a high serum T3/rT3 ratio, and anaemia. Especially RTHα patients with mild mutations and when diagnosed at young age seem to benefit from LT4 treatment. Further studies on treatment options and clinical biomarkers, which reflect the effects of LT4 treatment in both TRα- and TRβ-expressing tissues, will be of great value.

Concluding remarks

TH transport and action are two key players involved in TH homeostasis. In the last decades, research on normal thyroid physiology and molecular mechanisms underlying thyroid diseases has provided crucial new insights into the role of this important hormone.

This thesis expands our knowledge on the molecular characteristics of the thyroid hormone transporters MCT8 and MCT10, by showing that both transporters have a greater effect on intracellular T3 availability at the cell periphery than at the cell nucleus due to the rapidly established equilibrium between uptake and efflux of T3. This observation underscores the biological relevance of the efflux function of MCT8, which contributes to the understanding of the distinct phenotypes in AHDS patients.

Furthermore, this thesis describes the involvement of liver-specific transporters NTCP, OATP1B1 and OATP1B3 in targeting lipid-lowering TH analogs such as eprotirome and GC-1 to the liver. The fact that TH analogs may be targeted to particular tissues by usage of specific transporters provides new perspectives for future therapeutical purposes. Here, it remains important to exclude the possibility that the identified transporters are present in other tissues than the target tissue.

Lastly, this thesis describes both the phenotype and genotype of various RTHα patients in detail, including the effects of levothyroxine treatment in patients with different mutations. These studies facilitate the identification of other RTHα patients and thus contribute to the development of new treatment possibilities.

In the next years, more pieces will be added to the big puzzle, completing our knowledge on thyroid function in health and disease including better therapeutic options for patient care.

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SUMMARY

SUMMARY

Thyroid hormone (TH) is important for normal development and cellular metabolism. The thyroid gland secretes predominantly the prohormone T4 ad to a lesser extent the bioactive hormone T3. Hypothyroidism, a deficiency of TH, may result to symptoms such as fatigue, constipation, cold intolerance and weight gain. Because of the importance of TH for the developing brain, hypothyroidism may lead to severe defects in motor and mental development as illustrated by patients with untreated congenital hypothyroidism. In contrast, an excess of thyroid hormone (hyperthyroidism) can cause agitation, heat intolerance and weight loss.

Cellular TH homeostasis is regulated at different levels: 1) TH transporters mediate transport of the hormones across the plasma membrane, 2) different deiodinating enzymes in the cytoplasm activate the prohormone T4 to the bioactive T3 (deiodinases type 1 and 2) and inactivate T4 and T3 by the conversion to the inactive metabolites rT3 and T2 (deiodinase type 3), 3) T3 binds to TH receptors (TRs) in the nucleus, which modulate the expression of T3-target genes. The intracellular availability of TH is dependent on adequate function of transporter proteins and deiodinating enzymes, while TRs mediate TH action. Under physiological conditions, these different levels of control can precisely modify TH cellular content and action. Changes in any of these key players of peripheral TH regulation can alter TH signaling, for example as a consequence of altered cellular circumstances. However, defects in these key players of local TH homeostasis may cause disease. This thesis presents studies in which TH regulation has been investigated under both normal and pathological conditions, with the main focus on TH transport and action.

Chapter 1 provides a general background of normal TH physiology. The synthesis and importance of TH are further elucidated. Particular attention is paid to cellular transport of TH by different TH transporters, TH metabolism by different deiodinases and TH action via TRs. Lastly, the general aims and outline of this theses are presented.

Chapter 2 reports on the well known TH transporters MCT8 and MCT10, which mediate TH transport across the plasma membrane but also facilitate TH efflux out of cells. The objective of this study was to investigate if MCT8 and MCT10 increase the availability of TH for the nuclear TRs rather than generate a rapid equilibrium between TH in- and efflux across the plasma membrane. The results suggest that MCT8 and MT10, by virtue of their bidirectional TH transport, have less effect on steady-state nuclear TH levels than on TH levels at the cell periphery where D3 is located. The presence of MCT8 and MCT10 increase TH metabolism by D3 without increasing TH action in the nucleus. By preventing cellar efflux, the TH binding protein CRYM appears to increase the nuclear availability of TH.

In chapter 3 and 4 the characteristics of different human TH transporters in liver (OATPs and NTCPs) are explored. In **chapter 3** we demonstrate that human NTCP is a highly effective transporter of the TH analog eprotirome. In mice, eprotirome is also transported by mouse Ntcp, however the pharmacokinetics of eprotirome were not affected by Ntcp deficiency.

In **chapter 4** we extend the repertoire of liver transporters and show that the transporters OATP1A2, OATP1B1, OATP1B3, and NTCP can transport the TH analog GC-1, but also T3 and T4, across the plasma membrane and thus enable the activation of the nuclear TRβ1 receptor. This may explain the liver-selective action of these TH analogs. In addition, these findings provide insights into how TH analogs may be targeted to different tissues depending on the presence of specific transporters in these tissues.

In **chapter 5** we describe the youngest patient identified with resistance to TH (RTHα) due to a D211G missense mutation in both TRα1 and TRα2, together with her father who is also affected. In 2012, the first patients with RTHα due to inactivating mutations in TRα were identified. These patients are characterized by growth retardation, variable motor and cognitive defects, macrocephaly and abnormal thyroid function tests. In addition to these symptoms, this very young girl (18 months) with the D211G mutation in TRα1 and TRα2 displayed severe axial hypotonia. Early levothyroxine (LT4) treatment dit not only ameliorate her muscle tone, but also improved her growth and development. In addition, we studied the consequences of the mutation for TRα1/2 receptor function *in vitro*. The missense mutation led to a decreased transcriptional activity of TRα1, which could be overcome by higher T3 levels. The mutation did not affect the function of TRα2, which already in normal conditions can not bind T3.

Chapter 6 describes a total of 10 RTHα patients with three different mutations in TRα (C380fs387X, R384H, A263S). This large case series enables us to report on the diversity in the RTHα phenotype and compare the phenotypes of both affected and non-affected individuals from a family with the same mutation. The most frequent RTHα characteristics within this group of patients were anemia, constipation and a delay in reaching one or more developmental milestones. In addition, patients showed high/high-normal (F)T3 and low/normal (F)T4 and rT3 levels with a normal TSH. Furthermore, we studied the effects of the mutations on the TRα1/2 receptor function *in vitro*.

In **chapter 7** we report on the role of TH and TRα in human erythropoiesis. Animal studies suggest an important role for TH and TRα in the maturation of erythroid progenitor cells. In addition, almost all RTHα patients have mild anemia, the pathogenesis of which is unknown. We show that different TRα mutations affect the balane between proliferation and differentiation of erythroid prgenitor cells in human erythropoiesis, which likely contributes to the anemia seen in RTHα patients.

In **chapter 8** we present an overview of all RTHα patients identified so far (28 cases from 15 different families). We discuss the variation in phenotypes of the different RTHα

patients and describe the consequences of the different mutations on the receptor function *in vitro*. Hereby, we try to establish phenotyope-genotype correlations and we report on the association of the severity of the phenoytpe with the location and type of mutation in *THRA*. Lastly, we describe the effects of levothyroxine treatment in patients with distinct mutations with different responses to therapy, depending on the severity of the mutation.

In **chapter 9** the results presented in this thesis are discussed in view of the current literature. The possible implications ot the findings are explored and further directions for future research in the light of this thesis are provided.

SAMENVATTING

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Schildklierhormoon (SKH) wordt geproduceerd door de schildklier, een vlindervormig orgaan gelegen in de hals dat voornamelijk het prohormoon T4 en in mindere mate het bioactieve hormoon T3 afscheidt. SKH is cruciaal voor de normale ontwikkeling van vrijwel alle weefsels in het lichaam. Een tekort aan SKH (hypothyreoïdie) resulteert in klinische symptomen als moeheid, obstipatie, koude-intolerantie en gewichtstoename. Daarentegen leidt een overschot aan SKH (hyperthyreoïdie) juist tot gejaagdheid, warmte-intolerantie en gewichtsverlies. Het belang van SKH voor de hersenontwikkeling van de foetus wordt tevens benadrukt door patiënten met onbehandelde congenitale hypothyreoïdie, die zich presenteren met ernstige verstandelijke en motorische beperkingen.

De werking van SKH wordt in de cel op verschillende niveaus gereguleerd: 1) in de celwand zorgen SKH transporters voor de beschikbaarheid van SKH in de cel, 2) in de cel zorgen verschillende enzymen (dejodases) voor de omzetting van het prohormoon T4 naar het bioactieve hormoon T3 (dejodase type 1 en 2) en de omzetting van T4 en T3 naar de inactieve metabolieten rT3 en T2 (dejodase type 3), 3) in de celkern bindt T3 aan SKH receptoren (TRs), die fungeren als transcriptiefactoren en de expressie van T3-gevoelige genen activeren of onderdrukken. Binnen de SKH homeostase reguleren transporters en dejodases dus de SKH concentratie in de cel terwijl de SKH receptoren van belang zijn bij de uiteindelijke SKH actie. Een goede interactie tussen SKH transporters, dejodases en kernreceptoren zorgt in normale omstandigheden voor een nauwkeurige SKH concentratie in de cel, aangepast naar de behoefte van het lichaam. Veranderingen in de activiteit van één of meerdere van deze schakels kunnen onderdeel zijn van aanpassingsmechanismen, ten gevolge van gewijzigde omstandigheden. Wanneer dit niet het geval is en er sprake is van een defect in één van deze schakels, kan dit resulteren in verschillende ziekteprocessen. In dit proefschrift worden studies beschreven waarin de verschillende niveaus van SKH homeostase onder normale en abnormale omstandigheden zijn onderzocht, met de nadruk op SKH transport en actie.

In **hoofdstuk 1** wordt een algemene achtergrond van de SKH fysiologie beschreven. De productie van SKH door de schildklier en het belang van SKH worden nader toegelicht. Vervolgens wordt er dieper ingegaan op het cellulaire transport van SKH door verschillende SKH transporters, het SKH metabolisme door de dejodases en de SKH actie via de kernreceptoren. Het hoofdstuk wordt afgesloten met de hoofdlijnen en doelstellingen van dit proefschrift.

Hoofdstuk 2 beschrijft een studie met de bekende SKH transporters MCT8 en MCT10. De in de celmembraan gelegen MCT8 en MCT10 zorgen voor SKH transport vanuit het bloed naar de cellen. Echter faciliteren deze transporters ook SKH efflux uit de

cellen. Het doel van deze studie was daarom te onderzoeken of MCT8 en MCT10 de beschikbaarheid van SKH voor de kernreceptoren verhogen of dat er eerder een equilibrium wordt bereikt tussen SKH in- en efflux. De resultaten suggereren dat MCT8 en MCT10, vanwege hun bidirectionele SKH transport, meer invloed uitoefenen op de SKH concentratie in het cytoplasma dan in de celkern, waar de SKH receptoren zich bevinden. Aanwezigheid van MCT8 en MCT10 zorgt voor een toegenomen SKH metabolisme door het type 3 dejodase in het cytoplasma maar niet voor meer SKH actie in de celkern. Echter, wanneer de SKH efflux wordt tegengegaan door toevoeging van CRYM (een SKH bindend eiwit in het cytoplasma), neemt de SKH concentratie in de kern wel toe.

In de hoofdstukken 3 en 4 worden de kenmerken van verschillende humane SKH transporters (OATPs en NTCPs) in de lever bestudeerd en onderling vergeleken. In **hoofdstuk 3** laten we zien dat de humane NTCP het SKH analogon eprotirome effectief transporteert. In muizen wordt eprotirome ook door muis Ntcp getransporteerd, echter wordt de farmacokinetiek van eprotirome niet beïnvloed door afwezigheid van Ntcp. In **hoofdstuk 4** breiden we het repertoire aan levertransporters uit en laten we zien dat de transporters OATP1A2, OATP1B1, OATP1B3, en NTCP in staat zijn om het SKH analogon GC-1, maar ook T3 en T4, over het plasmamembraan te transporteren en vervolgens de SKH receptor TRβ1 te activeren. Dit kan de leverselectiviteit van deze SKH analoga verklaren. Deze bevindingen geven tevens inzicht in hoe SKH analoga gericht gebruikt kunnen worden voor verschillende weefsels, op basis van de aanwezigheid van specifieke transporters in deze weefsels.

In **hoofdstuk 5** beschrijven we de jongste patiënt met schildklierhormoonresistentie (RTHα) door een D211G puntmutatie in de SKH receptoren TRα1 en TRα2 samen met haar vader, die dezelfde mutatie heeft. In 2012 zijn de eerste patiënten met RTHα geïdentificeerd. Deze patiënten worden gekenmerkt door groeiachterstand, mentale en motorische beperkingen, een vergrote schedelomtrek (macrocefalie) en abnormale SKH waarden in het bloed. Dit 18 maanden oude meisje, met de D211G mutatie in TRα1 en TRα2, had naast bovengenoemde symptomen voornamelijk last van spierzwakte (hypotonie) in haar ledematen. Behandeling met SKH (levothyroxine) verbeterde niet alleen deze spierzwakte maar versnelde ook haar groei en ontwikkeling. Tevens onderzochten we de gevolgen van deze mutatie voor de functie van TRα1 en TRα2 *in vitro*. De resultaten laten zien dat de gemuteerde receptor D211G-TRα1 in mindere mate T3 kan binden, wat overkomen kan worden door de T3 concentratie te verhogen. De mutatie had geen invloed op de functie van TRα2, die in normale omstandigheden al geen T3 kan binden.

Hoofdstuk 6 omvat een gedetailleerde fenotypering van 10 RTHα patiënten met 3 verschillende mutaties in TRα (C380fs387X, R384H, A263S). Dit grote aantal patiënten stelde ons in staat om de diversiteit binnen het RTHα fenotype te bestuderen en bin-

nen één familie met dezelfde mutatie de aangedane individuen te vergelijken met hun niet-aangedane familieleden. De meest voorkomende RTHα kenmerken binnen deze patiëntengroep waren bloedarmoede (anemie), obstipatie en vertraging in het bereiken van één of meer ontwikkelingsmijlpalen. Daarbij hadden de patiënten hoog/hoog-normale (F)T3 en laag/normale (F)T4 en rT3 concentraties bij een normaal TSH. Eveneens hebben we de effecten van de mutaties op de functie van TRα1 en TRα2 *in vitro* bestudeerd.

In **hoofdstuk 7** onderzoeken we de rol van SKH en de SKH receptor TRα in de aanmaak van rode bloedcellen (erytropoëse) bij mensen. Verschillende muizenstudies suggereren immers dat SKH en TRα een belangrijke rol spelen bij de rijping van rode bloedcellen (erytrocyten). Bovendien hebben bijna alle patiënten met RTHα een milde anemie, waarvan de oorzaak onbekend is. In deze studie tonen we aan dat verschillende TRα mutaties het rijpingsproces van erytrocyt voorlopercellen beïnvloeden, waarbij de balans tussen proliferatie en differentiatie van deze voorlopercellen verstoord is.

In **hoofdstuk 8** presenteren we een overzicht van alle tot op heden geïdentificeerde RTHα patiënten (28 patiënten uit 15 verschillende families). We beschrijven de variatie in het fenotype van de verschillende patiënten samen met de gevolgen van de verschillende mutaties voor de receptorfunctie *in vitro*. Hierbij proberen we fenotype-genotype correlaties te maken en beschrijven we de associatie tussen de ernst van het fenotype en de locatie en type van de mutatie in *THRA*. Tenslotte beschrijven we de verschillen in respons op levothyroxine therapie in patiënten met diverse TRα mutaties en de samenhang met de ernst van de mutaties.

In **hoofdstuk 9** worden de resultaten van dit proefschrift bediscussieerd en in een breder perspectief geplaatst aan de hand van de huidige literatuur. De mogelijke implicaties van de bevindingen worden verkend en suggesties voor verder onderzoek naar aanleiding van dit proefschrift worden aangedragen.

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ERASMUS MC PHD PORTFOLIO

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Research skills	**Year**	**Workload**
Basic and Translational Endocrinology, Rotterdam	2013	5 days
Instellingsgebonden Stralingshygiënische Regelgeving B, Rotterdam	2013	3 days
Xth SNP Course &Symposium, Rotterdam	2013	5 days
Biomedical English Writing Course for MSc and PhD-students	2015	4 days
Integrity in Science Course	2015	1 day

Clinical courses		
Rotterdamse Internistendag, Rotterdam	2013	1 day
Rotterdamse Internistendag, Rotterdam	2014	1 day
Rotterdamse Internistendag, Rotterdam	2015	1 day

Presentations (international meetings)	**Year**	**Type**
The regulation of brain type 3 deiodinase by thyroid hormone, Science days internal Medicine, Antwerp, Belgium	2014	Poster
Clinical phenotype due to a mutation in the thyroid hormone receptor alpha 1, BSGPE vergadering, Zaventem, Belgium	2014	Oral
Effects of early LT4 treatment in a patient with a mutation in TRa1/a2, ETA, Santiago de Compostela, Spain	2014	Oral
Abnormal thyroid hormone metabolism in patients with THRA mutations due to impaired expression of the type 3 deiodinase, ESPE, Dublin, Ireland	2014	Oral
The diverse phenotype of mutations in T3 receptor alpha (TRa), ESPE, Dublin, Ireland	2014	Poster
Mutations in the T3 receptor TRa: genotype-phenotype correlation, Science days internal Medicine, Antwerp, Belgium	2015	Poster
Diverse genotypes and phenotypes of T3 receptor TRa mutations, ITC 2015, Lake Buena Vista, Florida, USA	2015	Oral
Co-chair symposium "TH action and physiology", ITC 2015, Lake Buena Vista, Florida, USA	2015	Chair
The diversity in phenotypes of patients with T3 receptor TRa mutations, Science days internal Medicine, Antwerp, Belgium	2016	Poster
Anemia in patients with resistance to thyroid hormone alpha: a role of TRa in human erythropoiesis, ETA, Copenhagen, Denmark	2016	Oral

Presentations (national meetings)		
Mutations in the thyroid hormone receptor alpha 1, Vergadering Adviesgroep	2013	Oral

Groeihormoon, Utrecht		
The regulation of brain type 3 deiodinase by thyroid hormone, Dutch Endocrine Meeting, Noordwijkerhout	2014	Oral
The regulation of brain type 3 deiodinase by thyroid hormone, XVIIIth annual symposium of the Dutch Thyroid Research Foundation, Amsterdam	2014	Oral
The diverse geno- and phenotype of mutations in the T3 receptor TRa, Dutch Endocrine Meeting, Noordwijkerhout	2015	Oral
Resistance to thyroid hormone alpha: a diversity in genotypes and phenotypes, Nuclear Receptor Research Network Meeting, Leiden	2015	Poster
The diverse phenotype of patients with mutations in TRa, XIXth annual symposium of the Dutch Thyroid Research Foundation, Amsterdam	2015	Oral
Resistance to thyroid hormone due to defective thyroid receptor alpha: a variation in phenotypes, Dutch Endocrine Meeting, Noordwijkerhout	2016	Oral
*Resistance to thyroid hormone alpha: a diversity in genotypes and phenotype*s, 8th Nuclear Receptor Research Network Meeting, Leiden	2015	Poster
Resistance to thyroid hormone due to defective thyroid receptor alpha: a variation in phenotypes, Dutch Endocrine Meeting, Noordwijkerhout	2016	Oral

(Inter)national conferences	**Year**	**Workload**
Erasmus MC PhD-day, Rotterdam	2013	1 day
Annual meeting of the European Thyroid Association, Leiden	2013	4 days
Symposium schildklierziekten: een update voor de klinische praktijk, Rotterdam	2013	½ day
6th Nuclear Receptor Research Network Meeting, Utrecht	2013	1 day
Science days Internal Medicine, Antwerp, Belgium	2014	2 days
Dutch Endocrine Meeting, Noordwijkerhout	2014	2 days
XVIIIth annual symposium of the Dutch Thyroid Research Foundation, Amsterdam	2014	1 day
International workshop on Resistance to thyroid hormone, Madrid, Spain	2014	4 days
Annual meeting of the European Thyroid Association, Santiago de Compostela, Spain	2014	5 days
Annual ESPE Meeting, Dublin, Ireland	2014	3 days
Science days Internal Medicine, Antwerp, Belgium	2015	2 days
Dutch Endocrine Meeting, Noordwijkerhout	2015	2 days
XIXth annual symposium of the Dutch Thyroid Research Foundation, Amsterdam	2015	1 day
15th International Thyroid Congress, Lake Buena Vista, Florida, USA	2015	6 days
6th Nuclear Receptor Research Network Meeting, Leiden	2015	1 day
Science days Internal Medicine, Antwerp, Belgium	2016	2 days

Dutch Endocrine Meeting, Noordwijkerhout	2016	2 days
Annual meeting of the European Thyroid Association, Copenhagen, Denmark	2016	4 days

Teaching activities

Skills training on thyroid (dys)function, first year medical students	2013	3 days
Supervision of Biomedical Science (VU Amsterdam) student, Bachelor project	2015	16 weeks
Skills training on thyroid (dys)function, first year medical students	2015	5 days
Skills training on thyroid (dys)function, second year clinical technology students	2015	1 day
Skills training on thyroid (dys)function, first year medical students	2016	3 days

Grants

Travel Grant Annual ESPE Meeting 2014, Dublin, Ireland (500€)	2014
ETA Research Grant 2015 (together with Prof. R. Peeters, 20,000€)	2015
ETA Travel Grant 2015/ITC (1000€)	2015
ITC 2015 Trainee Grant	2015
Trust Fonds Grant for ITC 2015 (500€)	2015
ETA Travel Grant 2016 (500€)	2016

ACKNOWLEDGEMENTS

ACKNOWLEDGEMENTS

Het blijft tegenstrijdig: het laatste hoofdstuk, wat vrijwel altijd als eerste gelezen wordt. Tijdens mijn promotietraject heb ik met veel verschillende mensen kunnen samenwerken, wat een geweldige ervaring is geweest. Daarom wil ik in dit hoofdstuk alle mensen bedanken, die hebben bijgedragen aan de totstandkoming van de voorgaande hoofdstukken.

Allereerst mijn promotor, **Prof.dr. ir. T.J. Visser**. Beste Theo, allerbeste Theo, onderzoek doen onder jouw supervisie was één groot feest! 3,5 jaar zijn werkelijk voorbij gevlogen en dit komt doordat elke dag in het lab naast uitdagend en leerzaam, vooral ook heel erg leuk was. Je laagdrempeligheid, oprechte interesse, aanstekelijke enthousiasme, flauwe grappen en bewonderenswaardige danskunsten waardeer ik enorm, net als je kunde - of in ieder geval poging - om ook van minder fraaie resultaten chocolade te maken. Ontzettend bedankt voor deze hele mooie tijd!

Wat een voorrecht, om niet één maar twee promotoren te hebben. Geachte **Prof.dr. R.P. Peeters**, beste Robin, jij wist me tijdens ons eerste gesprek meteen te enthousiasmeren voor de schildklier. Jij laat als geen ander zien hoe je het klinische werk succesvol met de wetenschap kunt verenigen. Ik heb veel bewondering voor het feit dat jij zoveel balletjes hoog weet te houden en hierbij elke dag ook goed gestemd kunt zijn. Je wist me telkens uit te dagen om in mogelijkheden te blijven denken en nóg een stapje verder te gaan, waarvoor ik je erg dankbaar ben!

Naast twee promotoren, schittert ook mijn copromotor, **dr. M.E. Meima**. Beste Marcel, eindelijk staat het zwart op wit: "You are the best scientist, evah!" Zonder jou was de titel van dit proefschrift waarschijnlijk "A collection of errors in laboratory research" geworden. Jij wist mijn soms niet geheel correcte gepipetteer altijd (nét op tijd) in goede banen te leiden, al zei je na een half jaar wel dat je al voldoende "materiaal" had voor mijn blooper-film. Je stond altijd open voor vragen en wist vaak een pokerface op te zetten wanneer het niet "de meest briljante vraag" betrof. Je hebt me ontzettend veel laboratoriumtechnieken geleerd en daarnaast ook mijn kennis over fossielen vergroot. Mijn dank is groot!

Dear **Prof.dr. Chatterjee**, dear Krish, thank you very much for taking part in the reading committee of my thesis. Collaborating with you and your research group was always very inspiring. Hopefully we can continue joining forces and acquire new insights into RTHα research.

Geachte **dr. Boelen**, beste Anita, veel dank voor het plaatsnemen in de promotiecommissie en ook je hartelijkheid en gezelligheid tijdens congressen.

Geachte **Prof.dr. Mathijssen**, beste Ron, hartelijk dank voor de beoordeling van mijn proefschrift.

Geachte **Prof.dr. van Gelder**, beste Teun, bedankt voor de initiële introductie aan het Erasmus MC en het opponeren tijdens mijn verdediging.
Geachte **dr. Simonides**, beste Dop, hartelijk dank voor je deelname aan mijn verdediging, de samenwerking en je altijd oprechte interesse in ons werk.
Geachte **Prof.dr. Meijer**, beste Onno, veel dank voor het opponeren tijdens mijn promotie.
Een groot woord van dank komt ook toe aan **dr. Thamar van Dijk**. Beste Thamar, jij hebt me helemaal wegwijs gemaakt op de 10e verdieping van de celbiologie. Ik vond het ontzettend mooi om samen aan een ex-vivo TRα model te werken. Bedankt voor je geduld en alle kennis die je met me gedeeld hebt.

Graag wil ik ook alle artsen bedanken, met wie we hebben samengewerkt rondom de RTHα patiënten. **dr. Korcan Demir**, **Prof.dr. Dionisios Chrysis**, **dr. Carla Moran**, and **Prof.dr. Anna Tylki-Szymanska** thank you very much for the collaboration. It was a great honor and pleasure! **Dr. Paul van Trotsenburg**, bedankt voor de samenwerking.

Dan mijn paranimfen, **Lotte Saes** en **Shona Kalkman**. Bedankt dat jullie mijn paranimfen zijn! Lieve Lotte, het prikken van bloedgassen tijdens ons 1e co-schap was het begin van een vriendschap vol avonturen. Etentjes, feestjes, culturele reisjes en nog zo veel meer. Met jou is alles genieten! Mooi om te zien hoe jij je ontwikkelt tot een geweldig internist-oncoloog. Lieve Shona, ook wij leerden elkaar kennen tijdens de co-schappen, die we combineerden met een wekelijkse pizza-date. Hierna stortten we ons beide op de wetenschap, waarbij ik de eer had naast jou te staan tijdens je verdediging. Van zeer wetenschappelijk verantwoorde discussies tot politiek incorrecte grappen, met jou is de gespreksstof nooit op. Op naar nog veel meer hoogtepunten samen met jullie!

De collega's van het schildklierlab, die elke dag van mijn promotietraject tot een genoegen hebben gemaakt. Beste **dr. Visser**, beste Edward, bedankt voor je inspirerende en motiverende input tijdens de besprekingen van gezamenlijke projecten. Lieve **Chantal**, anderhalf jaar lang waren wij labmaatjes. Wat hebben we het leuk gehad! **Layal**, jij was altijd te porren voor een gezellige kop koffie én goed overleg. **Elske**, je deelde de kamer met Marcel, wat heeft voorkomen dat fossielen de tent over hebben genomen, veel dank daarvoor! **Elaine** en **Karn**, gezien jullie lessen Nederlands hoef ik dit niet in het Engels te schrijven. Elaine, bedankt voor je onvoorwaardelijke steun bij mislukte experimenten of stressvolle perioden. Karn, erg leuk dat jij verder gaat met het RTHα project. Ik kijk uit naar je nieuwe wetenschappelijke bevindingen. **Stefan**, **Tim** en **Marco**, mannen, dank voor jullie altijd kritische en geïnteresseerde kijk op mijn gepresenteerde data. **Ramona** en **Selmar**, ontzettend bedankt voor alle hulp en "nummertjes" voor DNA-sequencing. **Alies** en **Simone**, veel dank voor jullie hulp bij het inwerken op het lab. **Mirjana** and

Arjola, thanks a lot for all fun at conferences and good luck with your further studies. **Carolien**, **Stefania**, **Giulia**, **Zhong Li** en **Melitza**, bedankt voor de gezelligheid en jullie vele bijdragen aan de wetenschappelijke prestaties van onze groep.

Alle collega's van de 5e verdieping, hartelijk dank voor de fijne tijd en jullie behulpzaamheid. In het bijzonder, **Jeroen van Rooij**, beste Jeroen, sinds de organisatie van de "labdag 2014" ben ik je als een goede vriend gaan beschouwen. Bedankt voor je luisterende oor en ook altijd kritische blik ten aanzien van mijn onderzoeksprojecten en überhaupt, de wetenschap in bredere zin.
Ramazan Buyukcelik, bedankt voor al je hulp bij de DNA extractie van onze bloedsamples en **Martin Huisman**, voor al het materiaal wat ik van jou gebietst heb. **Hans van Toor**, bedankt voor alle schildklierhormoonbepalingen. **Prof.dr. de Rijke**, bedankt voor alle controle bloedsamples. **Dr. Schaaf**, beste Gerben, veel dank voor de gezelligheid tijdens het pipetteerwerk op de 10e verdieping. Ook de secretaresses, **Annelies** en **Eline**, dank voor jullie hulp bij de meest uiteenlopende vragen. Jullie weten ook werkelijk overal raad mee! Dit geldt zeker ook voor **Anneke Hokke**, ontzettend bedankt!

Collega arts-assistenten en specialisten in het Sint Franciscus Gasthuis, bedankt voor de fijne samenwerking. Ik kijk uit naar de komende jaren!

Liefste **vrienden**: zowel van geneeskunde als buiten de studie, voormalig huisgenootjes, middelbare schoolvriend(inn)en, sportmaatjes, buurmeisjes, en alle mensen die niet onder het bovenstaande vallen. Wat prijs ik mezelf toch gelukkig met zó veel fijne mensen om me heen! Al vragen jullie zich waarschijnlijk nog steeds af wat ik al die jaren nou écht in het lab heb gedaan, jullie begrip voor mijn liefde voor CELLEN was altijd voelbaar. Apart wil ik noemen: **Anne (Visser)**, **Anne-Maayke**, **Pauline**; van de Monza in Utrecht tot de Ushuaia in Ibiza, chickies, met jullie is het altijd feest! **Nicole**, mijn enige vriendinnetje zonder agenda, bedankt voor je onvoorwaardelijke vriendschap. **Chantal**, **Aline** en **Jolien**, bedankt voor de eindeloze gezelligheid.

Lieve (Belgische) familie, lieve **oma**, **tante**, **ooms**, **nichtjes**, **neefjes**, **achterneefjes** en **achternichtjes**. Ik blijf het bijzonder vinden dat we zo'n grote familie vormen met inmiddels 4 generaties bij elkaar. Al lukt het me niet om alle feestdagen aanwezig te zijn, de keren dat ik er bij ben, geniet ik met volle teugen! In het bijzonder gaat mijn dank uit naar mijn talentvolle nichtje **Emma Anquinet**, die de cover van dit proefschrift heeft ontworpen.
Schoonfamilie. Lieve **Neil**, **Margot**, **Ruaridh**, **Colin** en **Astrid**, hartelijk dank voor alle mooie momenten samen en jullie altijd oprechte betrokkenheid. Op naar nog veel mooie jaren samen!

Dat laatste geldt natuurlijk ook voor **Duncan**. Stiekem weet iedereen dat jij een beetje met me mee bent gepromoveerd. Ik kan je niet voldoende bedanken voor je hulp en onvoorwaardelijke steun… áltijd!
Liefste (Russische) **opa** en **oma**, ontzettend bedankt voor jullie continue nieuwsgierigheid naar mijn werk en leven. Jullie interesse in én kennis van ontelbaar veel zaken is bewonderenswaardig, net als jullie plezier in het leven!
Lieve broeder, lieve **Philippe**. Je blijft altijd mijn kleine broertje, al ben je anderhalf keer zo lang als ik. Ik kijk naar je op, niet alleen omdat je boven me uitsteekt, maar vooral omdat je je ontwikkelt tot een hele fijne jongeman. Wacht maar tot jij eens gaat promoveren, kunnen we lachen!
Lieve ouders, lieve **mama** en **papa**. Mijn grootste dank gaat uit naar jullie. Voor de motivatie en stimulatie die jullie me altijd hebben gegeven, welke richting ik ook op wilde gaan. Jullie dagen me uit te streven naar het maximale en nooit voortijdig op te geven. Ik draag dit proefschrift dan ook op aan jullie!

ABOUT THE AUTHOR

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Anna Liya Maria van Gucht was born in 1988 in Moscow (Russia) and moved to Brussels (Belgium) at the age of four. In 2006 she graduated (cum laude) from the Stedelijk Gymnasium Nijmegen and she started studying Medicine at the University of Utrecht. During her clinical internships she worked two months at the gynecology department of Tygerberg Hospital in Cape Town (South Africa) and she did two months of research at the emergency department of Hopital Fernandez in Buenas Aires (Argentina). In February 2013 she received her medical degree and in April 2013 she started her PhD-project at the thyroid laboratory of the Erasmus Medical Center, headed by Prof.dr. Theo Visser and Prof.dr. Robin Peeters. The results of her 3.5 years of research are presented in this thesis. In October 2016 she started her training residencies in Internal Medicine at the Sint Franciscus Gasthuis, Rotterdam under supervision of Dr. Rietveld and Dr. Schrama.