Inter-individual differences in thyroid hormone bioactivity:

the effect of genetic variation

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Inter-individual Differences in Thyroid Hormone Bioactivity:

the effect of genetic variation

Inter-individuele verschillen in schildklierhormoon bioactiviteit:
het effect van genetische variatie

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Daglicht
Uit chaos van lakens en
voorgevoel opgestaan, gordijnen
open, de radio aan, was
plotseling Scarlatti
heel helder te verstaan:
Nu alles is zoals het is geworden,
nu alles is zoals het is
komt het, hoewel, misschien
hoewel, tenslotte nog in orde.

Judith Herzberg

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Chapter 1

General Introduction

Part of this chapter is based on

Genetic variation in thyroid hormone transporters

Wendy M. van der Deure, Robin P. Peeters and Theo J. Visser

Best Pract Res Clin Endocrinol Metab 2007 21:339-50

INTRODUCTION

Adequate levels of thyroid hormone are essential for normal development and growth, since thyroid hormone plays an important role in virtually all metabolic processes in the human body (1). This is clearly demonstrated in patients with thyroid hormone disorders. Hyperthyroidism leads to high circulating serum thyroid hormone levels. Patients complain of palpitations, excessive sweating, weight loss and can display swelling of thyroid gland, known as goiter (2-4). In contrast, decreased serum thyroid hormone levels due to hypothyroidism can result in weight gain, depression, atherosclerosis and hypertension (2, 5, 6).

Even subtle changes in serum thyroid parameters in patients with subclinical thyroid disease can have important consequences on thyroid hormone related end-points, such as atherosclerosis, heart rate, depression and osteoporosis (7-10). Therefore, it is likely that small variations in genes involved in thyroid hormone metabolism that result in altered thyroid hormone bioactivity can also have effects on clinical end-points. This thesis focuses on the effect of thyroid hormone pathway genes on serum thyroid hormone levels and clinical end-points using a candidate gene approach. In addition, associations between serum thyroid parameters and clinical endpoints, such as osteoporosis and hypertension are studied.

THYROID HORMONE SYNTHESIS AND THE HYPOTHALAMUS-PITUITARY-THYROID AXIS

The thyroid gland is situated in front of the thyroid cartilage in the neck. It contains two lobes joined by an isthmus and weighs only 20 grams in adults (11). It produces two hormones, i.e., thyroid hormone and calcitonin. The latter is secreted by the C-cells and is involved in the regulation of Ca^{2+}-homeostasis together with parathyroid hormone secreted by the parathyroid glands (11). The production of thyroid hormone occurs in the follicular cells and involves several steps, which start with trapping of iodide from the blood and the synthesis of thyroglobulin. This is followed by the production of monoiodotyrosyl and diiodotyrosyl residues, which are coupled to form thyroid hormone. Finally, thyroid hormone is released into the circulation by proteolysis of thyroglobulin (12). Under normal conditions, the main product secreted by the thyroid gland is the prohormone thyroxine (T4). Besides T4, a small amount of the active hormone 3,3',5-triiodothyronine (T3) is produced.

The production of thyroid hormone by the thyroid gland is regulated by the hypothalamus-pituitary-thyroid (HPT) axis (11) (Fig. 1). Thyroid hormone is secreted in response to thyroid-stimulating hormone (TSH), which is synthesized in and released from the pituitary and mediates its effect via binding to the TSH receptor (TSHR). In turn, TSH production is stimulated by hypothalamic thyrotropin releasing hormone (TRH). The production of TRH and TSH can be downregulated by thyroid hormones, a process known as negative feedback

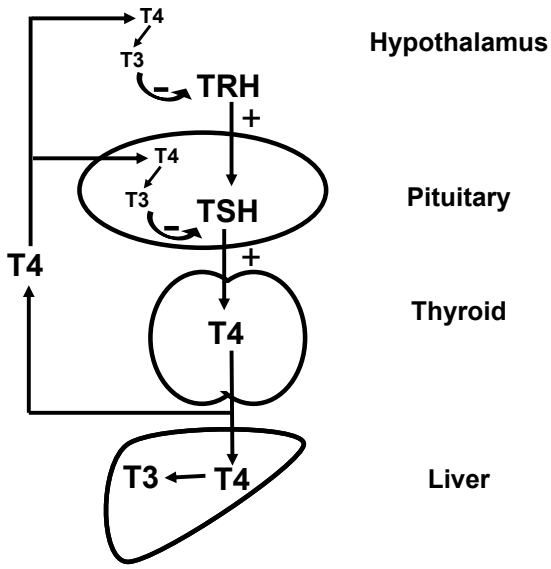

Figure 1: The hypothalamic-pituitary-thyroid axis.

regulation. However, also other hypothalamic hormones and drugs, such as somatostatin and bromocriptine can lower TSH production. Besides the regulation by TSH, thyroid hormone synthesis depends on the availability of iodine.

THYROID HORMONE ACTION AND METABOLISM

T3 exerts its effect by binding to nuclear thyroid hormone receptors (TR), thereby altering the transcription-rate of T3-responsive genes (13). The *THRA* and *THRB* genes encode TRα and TRβ, respectively. Both receptors exist in several isoforms, which arise from alternative splicing and the use of different transcription start sites (14-16). TRα1 appears at its highest levels in heart and brain, whereas TRβ1 is predominantly expressed in liver, kidney and thyroid (17). TRβ2 is important for regulation of the negative feedback of the HPT-axis (18). Since the TRs are located in the cell nucleus, the biological activity of thyroid hormone is mainly determined by the intracellular T3 concentration. This intracellular concentration is dependent on the circulating T3 concentration, transport of thyroid hormone across the cell membrane and the presence of iodothyronine deiodinases, which activate or inactivate thyroid hormone. Three deiodinases have been cloned and characterized as homologous selenoproteins, meaning that they have a selenocysteine in their catalytic center. This selenocysteine is encoded by a UGA codon (19). Since UGA is usually recognized as a STOP codon, a selenocysteine insertion

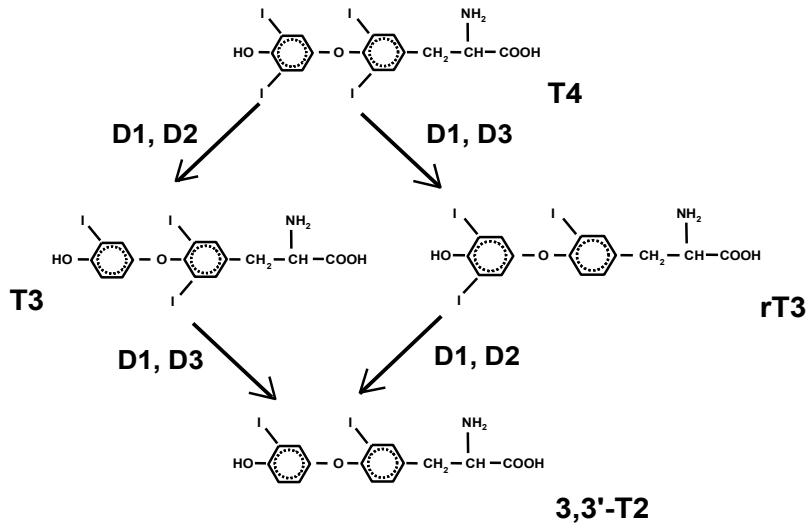

Figure 2: Structure of and relationship between the different iodothyronines, and their deiodination.

sequence (SECIS) element in the 3'untranslated region (3'UTR) of the deiodinase messenger RNA (mRNA) is necessary for selenocysteine incorporation at the site of a UGA codon.

Deiodinases are capable of outer- and inner ring deiodination (ORD and IRD respectively). ORD is the removal of an iodide atom at the phenol (outer) ring, whereas by IRD an iodide is removed from the tyrosyl (inner) ring. ORD is regarded as an activating pathway, whereas IRD is an inactivating pathway (Fig. 2).

Type I deiodinase (D1), expressed in liver, kidney and thyroid, facilitates ORD as well as IRD (19). The preferred substrate for D1 is the inactive metabolite reverse T3 (rT3) (20), which is converted to 3,3'-diiodothyronine (3,3'-T2) by ORD. This makes D1 the primary site for clearance of rT3. In addition, D1 is important for the production of plasma T3 from T4, although this conversion is much less efficient (21). D1 is under positive control of T3 and can be blocked by a drug commonly used in the treatment of hyperthyroidism, namely 6-propyl-2-thiouracil (PTU) (22).

Type II deiodinase (D2) is expressed in brain, pituitary, brown adipose tissue and vascular smooth muscle cells (19, 23). D2 has only ORD capacity and is thought to be important for local T3 production. However, recently Maia *et al.* demonstrated that D2 is present in skeletal muscle and could serve as a source of plasma T3 (24). D2 activity is increased in hypothyroidism, and decreased in hyperthyroidism.

Type III deiodinase (D3) is the major inactivating deiodinase, facilitating the conversion of T3 and T4 by IRD to 3,3'-T2 and rT3 respectively (19). D3 is expressed in placenta, brain, but also in fetal liver and various tumors (25-28). Its main function is to protect tissues from ex-

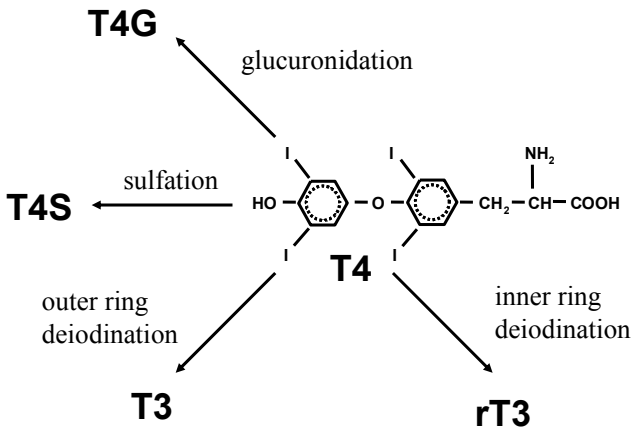

Figure 3: Pathways of thyroid hormone metabolism.

cess thyroid hormone, which is clearly demonstrated by Kester and colleagues, who showed in the human developing brain that D3 protects specific brain regions from excessive T3 until differentiation is required (29). On the other hand, high D3 activity in tumor tissues can lead to a state of consumptive hypothyroidism, meaning that the inactivation rate of thyroid hormone exceeds the production rate (28).

In addition to deiodination, thyroid hormones can also undergo other metabolic reactions, such as sulfation and glucuronidation (30) (Fig.3). Glucuronidation increases the water solubility of thyroid hormones, thereby facilitating excretion in the bile (31). Sulfation is catalyzed by a group of cytosolic sulfotransferases (SULTs), located in different tissues such as the liver, kidney and brain (32). All members of the human SULT1 family catalyze the sulfation of thyroid hormone (33-36). The physiological role of sulfated iodothyronines is not completely clear. The main function of sulfation might be the irreversible degradation of thyroid hormone (30). It has, however, also been suggested that sulfates represent a reservoir of inactive thyroid hormone, from which active thyroid hormone is recovered when required (37). The serum concentrations of sulfated iodothyronines, such as T4 sulfate (T4S) and T3 sulfate (T3S), are low under normal conditions due to rapid deiodination by D1 (30, 38-41). However, sulfated iodothyronines become elevated in preterm infants or during critical illness (39, 41, 42).

THYROID HORMONE TRANSPORT

Thyroid hormones circulate in serum bound to the carrier proteins thyroxin binding globulin (TBG), transthyretin (TTR) and albumin. In humans TBG, TTR and albumin carry about 75%,

15% and 10% respectively, of T4 and T3 (20, 43, 44). Therefore, serum free T4 (FT4) and free T3 (FT3) only comprise 0.02% and 0.2% of total T4 and T3 (43-45). The free fraction is responsible for thyroid hormone action. However, since both TRs and deiodinases are located intracellularly, transport of thyroid hormone across the cell membrane is required. Based on the lipophilic structure of thyroid hormones, it was assumed that thyroid hormone enters the cell through passive diffusion. However, it has become increasingly clear that there are specific thyroid hormone transporters, and that the activity of these transporters in part determines the intracellular thyroid hormone concentration (45).

Recently, the monocarboxylate transporter 8 (MCT8) has been characterized as an active and specific thyroid hormone transporter (46). Especially the finding that mutations in the *MCT8* gene result in severe mental retardation and elevated serum T3 levels, also known as the Allan-Herndon-Dudley syndrome, led to great scientific interest in thyroid hormone transport (47, 48). In this thesis, organic anion transporting polypeptides (OATPs) have been studied as candidates for thyroid hormone transport. Therefore, other proteins capable of thyroid hormone transport, such as Na^+/taurocholate co-transporting polypeptide (NTCP) (49), L-type amino acid transporter (LAT) (50) and the MCTs (46, 51) will not be discussed in this introduction.

Organic anion transporting polypeptides

The OATPs are a large family of transporters responsible for Na^+-independent transmembrane transport of amphipathic organic compounds, including bile salts, bromosulfophthalein (BSP), steroid hormones and numerous drugs (52). So far, 11 OATPs have been identified in humans, whereas in mice and rats respectively 13 and 14 members have been found (Fig. 4).

All OATPs are proteins of 652–848 amino acids in length with 12 transmembrane domains. Most OATP proteins are expressed in multiple tissues, including liver, kidney, brain (blood–brain barrier, choroid plexus), lung, heart, placenta, testis, eye, and small intestine (53). However, some members of the OATP family show a tissue-specific distribution; OATP1B1 and OATP1B3 are exclusively expressed in liver (54, 55), whereas OATP1C1 is only present in the brain and in the Leydig cells of the testis (56). Furthermore, most OATP family members are expressed at the basolateral membrane of polarized cells (54, 55, 57).

OATPs are generally involved in transport of both endo- and xenobiotics. Among the many ligands transported by OATPs, several members of this large family also facilitate uptake of iodothyronines. These include members of the OATP1 subfamily: OATP1A2 (58, 59), OATP1B1 (58, 60), OATP1B3 (58, 61) and OATP1C1 (56); members of the OATP4 subfamily: OATP4A1 (59) and OATP4C1 (62); and a member of the OATP6 subfamily: OATP6C1 (63). Interestingly, all members of the OATP1 subfamily that transport iodothyronines, form a gene cluster together with a related pseudogene on human chromosome 12p and are highly homologous to each other (Fig. 5).

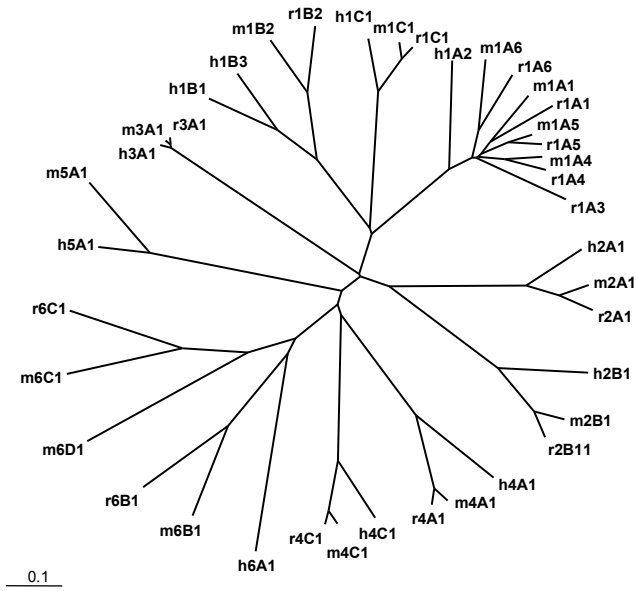

Figure 4: Phylogenetic tree of the OATP family.

OATP1A2, first cloned and characterized in 1995 as a transporter for bile salts and BSP in human liver by Kullack-Ublick (64), has been shown to transport T3 and T4 with Km values of 7 and 8 μM, respectively (59). Furthermore, both OATP1B1 and OATP1B3 are involved in uptake of thyroid hormone into the liver, besides the transport of bile salts, various drugs, such as HMG-CoA-reductase inhibitors and antibiotics (54, 65, 66). Like the previous OATPs, also OATP4A1, OATP4C1 and OATP6C1 facilitate transport of iodothyronines among a wide spectrum of ligands (67). A notable exception to the multi-specific transport capacity of OATPs is OATP1C1, previously known as OATP-F. OATP1C1 has been shown to be a high affinity transporter for T4 and rT3, but not T3 (56). Based on its expression in capillaries throughout the brain and its high affinity for T4, OATP1C1 is thought to be important for uptake of T4 into the brain across the blood-brain barrier.

VARIATION IN SERUM THYROID HORMONE LEVELS IN THE NORMAL POPULATION

In healthy subjects, serum thyroid parameters show substantial inter-individual variability, whereas the intra-individual variability is within a narrow range (68). Together with environmental factors such as diet or smoking, genetic factors are thought to contribute significantly

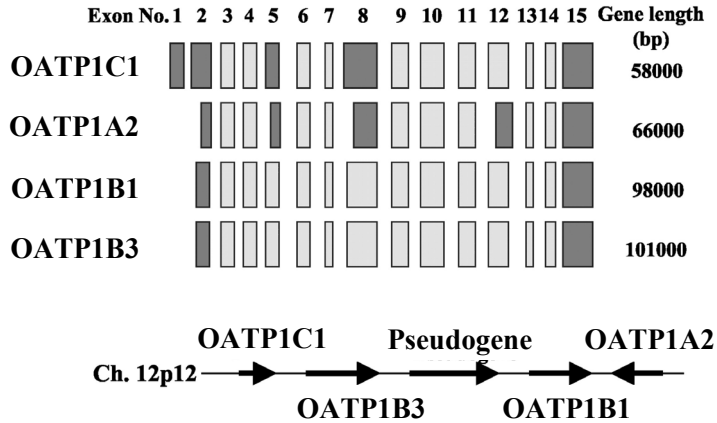

Figure 5: Genomic organization of OATP1C1, OATP1A2, OATP1B1 and OATP1B3 and the genomic structure of the human OATP-cluster located on chromosome 12p12 (adapted from (56)).
Light gray blocks represent exons with identical length, and dark gray blocks represent exons with different lengths for the indicated genes.

to this inter-individual variability, resulting in a thyroid function set-point that is different for each individual. This hypothesis is supported by a classical twin study, in which heritability accounted for ~ 65% of the variation in serum TSH, FT4 and FT3 levels (69). In a Mexican-American population, total heritability in serum thyroid parameters ranged from 35–64% of the total inter-individual variation observed (70). Slightly different numbers were found in a study of a large UK twin cohort (71) (Table 1).

Genetic association studies

Approximately 99.9% of the human genome is identical between individuals. The remaining 0.1% DNA sequence variation is in part responsible for many individual phenotypic characteristics, such as eyecolour or haircolour, but also a person's risk to develop complex diseases such as coronary heart disease, diabetes or cancer, or the response to treatment (72-77). Such

Table1: Estimates of the genetic contribution to the variation in serum thyroid hormone levels from different studies

	Proportion of variance in serum thyroid hormone levels attributable to genetic effects		
	Hansen et al. (69)	Samollow et al. (70)	Panicker et al. (71)
TSH	64%	37%	65%
FT4	65%	35%	39%
FT3	64%	64%	23%

genetic variations also contribute to the inter-individual variability in serum thyroid parameters (78-80). The most commonly observed and studied variations in the human genome are single nucleotide polymorphisms. Polymorphisms are variations in the nucleotide sequence of the genome that occur in at least 1% of the general population. Other forms of variations are variable number of tandem repeat polymorphisms and deletion/insertion polymorphisms.

Different approaches can be used to study the contribution of polymorphisms to the phenotype of interest. However, first of all, polymorphisms need to be identified. This can be done by sequencing the entire gene of interest in a number of subjects. Although the amount of information generated by this method is impressive, it is very time-consuming and costly. Nowadays, more and more information is available on the Internet, in databases such as the HapMap Project (www.hapmap.org) and the National Center for Biotechnology Information dbSNP database (www.ncbi.nlm.nih.gov) (81). Information from sequencing efforts is collected in dbSNP, a database containing information on all common genetic variants, which currently are estimated to amount to ~15 million polymorphisms. The Hapmap Project is a multi-country effort to identify and catalog genetic similarities and differences in humans. On their website, genotype information from 270 individuals from 4 geographically diverse populations can be found.

Once polymorphisms have been identified, different approaches can be used to study the contribution of polymorphisms to a phenotypic trait. With a candidate gene approach, genes are selected based on their biological role (82). For instance, polymorphisms in D1 are likely to be associated with serum thyroid parameters since D1 converts T4 to T3. Subsequently, the most promising polymorphisms are chosen, such as those that lead to an amino-acid change in the encoded protein, e.g., non-synonymous polymorphisms. Other interesting polymorphisms are located in the promoter region and in the 3' untranslated region (3'UTR), since these may modify mRNA expression or stability. In addition, due to linkage disequilibrium adjacent polymorphisms tend to be inherited as a block, a so-called haplotype block. A haplotype block may contain a large number of polymorphisms, yet only a few are sufficient to identify most polymorphisms in such a block due to high linkage disequilibrium. These specific polymorphisms are called tagging polymorphisms.

The next step in the assessment of the contribution of a polymorphism to a phenotypic trait involves the characterization of the phenotype, such as serum TSH or osteoporosis, and genotyping of a representative population (83). In the last years, Peeters and colleagues have identified several polymorphisms in thyroid hormone pathway genes and associated them with serum thyroid parameters. For instance, the T allele of the D1-C785T polymorphism is associated with higher serum FT4 and rT3 levels (78). In addition, carriers of the TSHR-Glu^{727} allele have lower serum TSH levels than non-carriers (78). These polymorphisms do not only influence serum thyroid parameters, but are also associated with thyroid hormone related clinical endpoints. For instance, the TSHR-Asp727Glu polymorphism is associated with insulin resistance in elderly men (84).

Currently, an increasing number of studies use a hypothesis-free approach instead of a candidate gene approach by performing a genome-wide association (GWA) analysis. In such a GWA study the genome of each individual in the population is typed for more than 500.000 polymorphisms to search for variants that are associated with the phenotype of interest. Most genetic association studies performed in this thesis are based on a candidate gene approach, while chapter 10 shows the results of a pathway analysis of genes involved in thyroid hormone synthesis, metabolism and transport using GWA data of the Rotterdam Study.

Populations used for genetic association studies

For many of the genetic association studies, a population of 155 healthy blood donors was used as a 'training' cohort (78). A number of the studies were conducted within the Rotterdam Study, a population-based cohort study of 7983 men and women aged 55 years and older and living in a well-defined suburb of Rotterdam, the Netherlands (85). In addition, the Rotterdam Scan Study is an ongoing prospective population-based cohort study designed to study causes and consequences of age-related brain changes on MRI (86). Some studies were part of a nationwide project (GEMINAKAR) investigating the relative influence of genetic and environmental factors on a variety of different traits among Danish twins (87). Finally, a cohort of patients, between 18 and 70 years of age, who had been on an adequate dose of LT4 replacement therapy as reflected by normalized serum TSH levels for primary autoimmune hypothyroidism for at least 6 months was used. The patients participated in a randomized controlled trial investigating possible superiority of therapy with a LT4/LT3 combination in a ratio of 5:1 or 10:1 over therapy with LT4 alone (88).

AIM AND OUTLINE OF THIS THESIS

Subtle changes in serum thyroid parameters have important consequences on thyroid hormone related endpoints, such as atherosclerosis, heart rate, depression and osteoporosis (7-10). Therefore, the aim of this thesis was to determine the effect of polymorphisms in genes involved in thyroid hormone metabolism and transport on serum thyroid hormone levels and clinical endpoints, and to associate serum thyroid parameters with thyroid hormone related endpoints.

In the first part of this thesis several members of the OATP family were characterized for thyroid hormone transport. If these transporters were found to transport thyroid hormone significantly, a candidate gene approach was used to study the physiological relevance of the transporter of interest. In chapter 2 OATP1B1 was studied in relationship to serum thyroid parameters and estrogen levels. In chapter 3 and 4 the effect of genetic variation in OATP1C1 on serum thyroid parameters and depression is discussed, whereas chapters 5 deals with OATP1A2 and OATP1B3.

In chapter 6 we studied the contribution of the TSHR-Asp727Glu polymorphism to the variance in serum TSH levels. In chapter 7 the relationship between serum thyroid hormone levels, the TSHR-Asp727Glu polymorphism and bone parameters was analyzed. Chapter 8 focuses on the *in vivo* and *in vitro* effects of polymorphisms in D1. Chapter 9 deals with associations between serum thyroid parameters, polymorphisms in D2 and blood pressure and the occurrence of hypertension. This thesis ends with chapter 10, which shows the results of a pathway analysis of genes involved in thyroid hormone synthesis, metabolism and transport using GWA data of the Rotterdam Study. Finally, in chapter 11 all results are discussed and put in a broader perspective in the general discussion.

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Chapter 2

OATP1B1: an important factor in hepatic thyroid hormone and estrogen transport and metabolism

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ABSTRACT

Sulfation is an important pathway in the metabolism of thyroid hormone and estrogens. Sulfation of estrogens is reversible by estrogen sulfatase, but sulfation of thyroid hormone accelerates its degradation by the type I deiodinase (D1) in liver. Organic anion transporters are capable of transporting iodothyronine sulfates such as T4 sulfate (T4S), T3 sulfate (T3S) and reverse T3 sulfate (rT3S) or estrogen sulfates like estrone sulfate (E1S), but the major hepatic transporter for these conjugates has not been identified. A possible candidate is OATP1B1 since model substrates for this transporter include the bilirubin mimic bromosulfophthalein (BSP) and E1S, and it is highly and specifically expressed in liver. Therefore, OATP1B1-transfected COS1 cells were studied by analysis of BSP, E1S and iodothyronine sulfate uptake and metabolism. Two Caucasian populations (155 blood donors and 1012 participants of the Rotterdam Scan Study) were genotyped for the OATP1B1-Val174Ala polymorphism and associated with bilirubin, E1S and T4S levels.

OATP1B1-transfected cells strongly induced uptake of BSP, E1S, T4S, T3S and rT3S compared to mock transfected cells. Metabolism of iodothyronine sulfates by co-transfected D1 was greatly augmented in the presence of OATP1B1. OATP1B1-Val^{174} showed a 40% higher induction of transport and metabolism of these substrates than OATP1B1-Ala^{174}. Carriers of the OATP1B1-Ala^{174} allele had higher serum bilirubin, E1S and T4S levels.

In conclusion, OATP1B1 is an important factor in hepatic transport and metabolism of bilirubin, E1S and iodothyronine sulfates. OATP1B1-Ala^{174} displays decreased transport activity and thereby gives rise to higher bilirubin, E1S and T4S levels in carriers of this polymorphism.

INTRODUCTION

Sulfation is an important pathway in the metabolism of different hormones, such as thyroid hormone and estrogens. This reaction is catalyzed by a group of cytosolic sulfotransferases (SULTs) located in different tissues such as the liver, kidney and brain (1). All members of the human SULT1 family catalyze the sulfation of thyroid hormone (2-5). Sulfation of estrogen is, however, mainly carried out by SULT1E1 (1).

The serum concentrations of sulfated iodothyronines, such as T4 sulfate (T4S), T3 sulfate (T3S) and reverse T3 sulfate (rT3S), are low under normal conditions as they are rapidly degradated by the type 1 deiodinase (D1) (6-11). However, serum iodothyronine sulfate levels are high in preterm infants and during critical illness, possibly due to a decreased metabolism (7, 9, 12). Serum concentrations of estrone sulfate (E1S) are 10-20 times higher than those of the unconjugated estrogens, thereby reflecting the primary role of sulfation in estrogen metabolism (13). The formation of E1S serves as a reservoir for active estrogen, since the sulfation of estrogens is readily reversible by estrogen sulfatase (14).

Different organic anion transporters (OATPs) are capable of transporting iodothyronine and estrogen sulfates (15-17), but the major hepatic transporter for these conjugates has not yet been identified. A possible candidate is OATP1B1 since model substrates for this transporter include the bilirubin mimic bromosulfophthalein (BSP) and E1S, and it is highly and specifically expressed in liver (16, 18). Therefore, OATP1B1-transfected COS1 cells were studied by analysis of BSP, E1S, T4S, T3S and rT3S uptake and metabolism.

Recently, various polymorphisms in the *SLCO1B1* gene, encoding OATP1B1, have been identified (19). Especially, the OATP1B1-Val174Ala polymorphism is of interest, since the valine at position 174 is conserved in all members of the OATP1 subfamily and is located in a putative transmembrane region of OATP1B1, which is important for substrate recognition and transport (20) (Fig. 1). In addition, this polymorphism is associated with higher serum bilirubin levels due to an altered transport function of the protein (21). We, therefore, analyzed the effect of this polymorphism on the function of OATP1B1 *in vivo* and *in vitro,* as a transporter for sulfated iodothyronines and estrogens.

MATERIALS AND METHODS

Constructs

The pCMV6-XL4-OATP1B1 plasmid was kindly provided by Prof. Dr. Peter J. Meier (Institute of Clinical Pharmacology and Toxicology, University Hospital Zürich, Switzerland). The Val174Ala polymorphism was introduced into the OATP1B1 expression vector by site-directed mutagenesis according to the Quick Change site-directed mutagenesis protocol of Stratagene. The sense and antisense primers containing the nucleotide change needed to produce

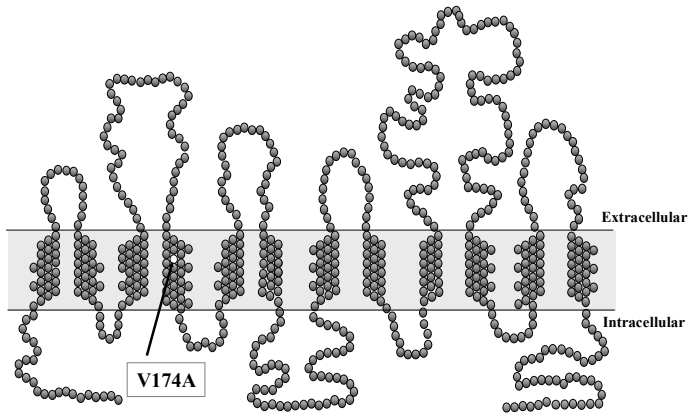

Figure 1: A putative two-dimensional model of the human OATP1B1 protein with localization of valine to alanine amino acid change caused by the Val174Ala polymorphism in the *SLCO1B1* gene (Chr 12p12) (adapted from (19)).

the OATP1B1-Ala^{174} variant (sense 5'-CTG GGT CAT ACA TGT GGA TAT ATG CGT TCA TGG GTA ATA TGC-3') were used in circular mutagenesis reactions with 100 ng plasmid template and 1 U of Pfu DNA polymerase. The construct with the OATP1B1-Val174Ala polymorphism was fully sequenced on an automated ABI 3100 capillary sequencer, using the Big Dye terminator cycle sequencing method (Applied Biosystems) to verify that the desired mutation had been generated, and that no other mutations had occurred during amplification.

Human μ-crystallin (CRYM) cDNA was purchased from the RZPD German Resource Center for Genome Research (www.rzpd.de) and subcloned into pSG5 (Stratagene) using *Eco*RI and *Bam*HI sites. pcDNA3-hMCT8 and pcDNA3-ratD1 (rD1) plasmids were constructed as previously described (22).

Cell culture

COS1 cells were cultured in 6 or 24-well dishes (Corning, Schiphol, The Netherlands) with DMEM/F12 medium (Invitrogen), containing 9% heat-inactivated fetal bovine serum (Invitrogen) and 100 nM sodium selenite (Sigma).

BSP, E1S and iodothyronine (sulfate) transport and metabolism by OATP1B1

Materials: FuGENE6 transfection reagent was obtained from Roche Diagnostics (Indianapolis, USA). [^{125}I]NaI, [^{125}I]T4 and [^{125}I]T3 were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). [^{125}I]rT3 and [^{3}H]E1S were purchased from Perkin Elmer (Boston, USA) and unlabeled T4, T3, rT3 and E1S from Henning GmbH (Berlin, Germany). BSP was obtained from Fluka Chemica (Buchs, Switzerland). [^{125}I]BSP was prepared by radioiodination of BSP

using the chloramine-T method, and purified on a small (~1 ml bed volume) Sephadex LH20 column by successive rinsing with 0.1 M HCl and water, and final elution with 0.1 M NH4OH in ethanol. The purity of [^{125}I]BSP was checked by HPLC analysis. For [^{125}I]T4S, [^{125}I]T3S and [^{125}I] rT3S synthesis, solutions of [^{125}I]T4, [^{125}I]T3 or [^{125}I]rT3 were evaporated to complete dryness under a stream of N2. 0.2 ml ice-cold chlorosulfonic acid in dimethylformamide (1:4, v/v) was added to the residues. The mixtures were incubated for 2 h at 37 C, and the reactions were stopped by adding 0.8 ml ice-cold water. Sulfates thus synthesized were separated on a Sephadex LH-20 column (23). The final purified preparations contained virtually no contamination from free iodide or native hormone as checked by HPLC analysis.

Uptake studies: COS1 cells were cultured in 6-well culture dishes, and transfected in duplicate with 500 ng pCMV6-XL4 plasmid without or with OATP1B1-Val^{174} or Ala^{174} insert using FuGENE6 according to the manufacturer's guidelines. After 36 h, cells were washed with incubation medium (Dulbecco's PBS, 0.1% BSA and 0.1% glucose), and incubated for 5, 10 or 30 min at 37 C with 1 nM (2x10^5 cpm) ^{125}I-labeled BSP, T4, T3, rT3, T4S, T3S or rT3S in 1.5 ml incubation medium. After incubation, medium was aspirated and cells were washed with incubation medium, lysed with 0.1 M NaOH and counted for radioactivity in a gamma scintillation counter. In the experiments with [^{3}H]E1S, cells were washed with incubation medium (Dulbecco's PBS with 0.1% glucose), and incubated for 1, 2, 5 or 10 min at 37 C with 100 nM [^{3}H]E1S in 1.5 ml incubation medium. Cells were lysed with 0.1% SDS and subsequently counted for radioactivity. Except for the experiments with [^{125}I]BSP and [^{3}H]E1S, uptake was determined in cells co-transfected with the high-affinity cytosolic thyroid hormone-binding protein CRYM to prevent efflux of internalized iodothyronines (24). For T4, T3 and rT3 uptake experiments, pcDNA3-MCT8 plasmid was used as positive control.

Metabolism studies: COS1 cells were cultured in 24-well culture dishes and transfected with 100 ng pcDNA3-rD1 and 100 ng pCMV6-XL4 plasmid without or with OATP1B1-Val^{174} or Ala^{174} insert. 36 h after transfection, cells were incubated for 24 h at 37 C with 1 nM (1x10^6 cpm) [^{125}I] T4S, T3S or rT3S in 0.5 ml incubation medium. After incubation, medium was harvested and analyzed by HPLC as described previously (22).

Study populations

Genotypes of the OATP1B1-Val174Ala polymorphism were determined in 158 healthy blood donors from the Sanquin Blood Bank South West region (Rotterdam, The Netherlands) (25). Informed consent was given by all donors.

Participants of the Rotterdam Scan Study, an ongoing prospective population-based cohort study designed to study causes and consequences of age-related brain changes on MRI (26), were also genotyped. Data on serum thyroid parameters and the use of thyroid medication were available in 1045 participants. E1S levels were determined in a subgroup

of 430 participants. The Rotterdam Scan Study was conducted in accordance with the tenets of the Declaration of Helsinki. The Medical Ethics Committee of the Erasmus University approved the study.

Serum analyses

Total bilirubin was determined by the Jendrassik-Grof diazo method on the Hitachi 912 (Roche Diagnostics, Germany), and E1S was measured by a RIA from DSL (Webster, Texas, USA). TSH, FT4 and T3 were measured by chemoluminescence assays (Vitros ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, Rochester, NY, USA). RT3 was measured with an in-house RIA (27), while T4S was determined by a specific RIA as previously described (6). T4S data were only available in healthy blood donors, whereas E1S levels were only determined in participants of the Rotterdam Scan Study.

Genotyping

DNA was isolated from peripheral leucocytes by standard procedures. The OATP1B1-Val174Ala (rs4149056) genotypes were determined by 5'fluorogenic Taqman assays. Reactions were performed in 384-wells format on ABI9700 2x384 well PCR machines with endpoint reading on the ABI 7900HT Taqman machine (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) (28). All healthy blood donors were successfully genotyped. In the Rotterdam Scan Study, DNA was available from 989 of the 1012 participants. The genotyping success rate was 98.8%, with 12 samples that failed genotyping.

Statistical analysis

Data were analyzed using SPSS 10.0.7 for Windows (SPSS, Inc., Chicago, IL). P values are two-sided throughout and were considered significant if <0.05.

For the *in vitro* experiments, unpaired Student's T-tests were used to test whether the iodothyronine uptake and metabolism induced by OATP1B1-Val^{174} was significantly different from that in cells transfected with OATP1B1-Ala^{174} or empty vector.

For the OATP1B1-Val174Ala polymorphism, deviation from Hardy-Weinberg equilibrium proportions was analyzed using a Chi-square test. Differences between genotypes were adjusted for age and gender and tested by analysis of covariance (ANCOVA). The analysis of the effect of the polymorphism on serum thyroid parameters was carried out after exclusion of subjects taking thyroid medication or any other drugs known to interfere with thyroid hormone metabolism and/or subjects with serum thyroid parameters indicating overt thyroid disease. Hypothyroidism was defined by a serum TSH concentration above the reference range (0.4-4.3 mU/l) and FT4 below the reference range (11-25 pmol/l). Hyperthyroidism was defined by serum TSH <0.4 mU/l and FT4 >25 pmol/l. Based on these exclusion criteria, 3 subjects were excluded in the population of healthy blood donors and 33 subjects in the Rotterdam Scan Study. Therefore, 155 healthy blood donors and 1012 subjects of the Rotterdam

Scan Study were eligible for analysis. For the analysis of the effect of the OATP1B1-Val174Ala polymorphism on E1S levels, subjects on estrogen drug therapy (n=6) were excluded, leaving 424 subjects eligible for analysis. Due to non-normal distribution, bilirubin, E1S, TSH and T4S levels were logarithmically transformed.

RESULTS

Transport of BSP, E1S, and (sulfated) iodothyronines by OATP1B1

Previously, BSP and E1S have been used as model substrates to characterize OATP1B1 (16, 18, 19). In line with results from earlier studies (16, 18, 19), uptake of BSP and E1S was strongly induced in cells transfected with OATP1B1 compared to mock transfected cells. Maximum uptake of E1S was already obtained after 1 min, pointing towards a rapidly reached equilibrium between uptake and efflux of E1S in a short time period. Subsequently, the percentage uptake remained the same even when cells were incubated for longer time periods.

Induction of BSP and E1S uptake was approximately 50% lower for the OATP1B1-Ala^{174} variant in comparison with the Val^{174} variant (Fig 2).

For uptake experiments with (sulfated) iodothyronines, cells were co-transfected with CRYM (24). This protein binds not only iodothyronines, but also sulfated iodothyronines (personal communication W.E. Visser). Co-transfection of cells with CRYM and OATP1B1 did not result in significant uptake of [^{125}I]T4 and [^{125}I]T3, in contrast to cells co-transfected with CRYM and MCT8, a very active T3 and T4 transporter (29) (Fig. 3). In addition, OATP1B1-Val^{174} and OATP1B1–Ala^{174} showed little transport of [^{125}I]rT3 in comparison to MCT8. However, uptake of the sulfated iodothyronines [^{125}I]T4S, [^{125}I]T3S and [^{125}I]rT3S was clearly induced in cells transfected with OATP1B1 (Fig. 4) compared to cells transfected with the empty vector. Induction of uptake of the iodothyronine sulfates was 50-70 % lower for the Ala^{174} variant than for the Val^{174} variant.

Iodothyronine sulfate metabolism in cells co-transfected with OATP1B1 and D1

T4S, T3S and rT3S metabolism by rD1 was markedly increased when cells were co-transfected with OATP1B1 (Fig. 5). About 20% of T4S and T3S was deiodinated by rD1 when OATP1B1-Val^{174} was co-transfected, whereas no iodide was produced in cells transfected with rD1 alone. Only 3% of rT3S was deiodinated in D1-transfected cells, whereas almost 60% of rT3S was deiodinated by D1 in the presence of OATP1B1. Induction of T4S, T3S and rT3S metabolism was 40 to 50% lower for the OATP1B1-Ala^{174} than for the Val^{174} variant (Fig. 5).

Association of OATP1B1-Val174Ala with serum bilirubin and hormone levels

Table 1 shows the characteristics of the population of blood donors and of the subjects participating in the Rotterdam Scan Study. In the cohort of healthy blood donors, 103 subjects

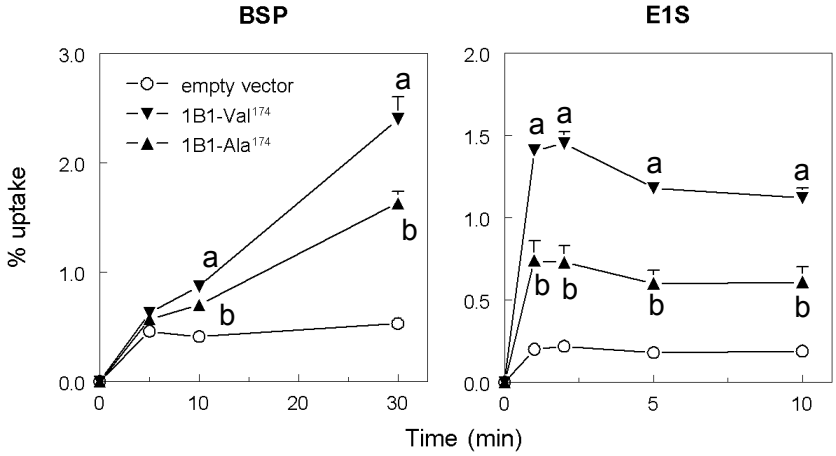

Figure 2: [^{3}H]BSP and [^{125}I]E1S uptake by COS1 cells transfected with empty vector, OATP1B1-Val^{174}, or OATP1B1-Ala^{174}. Cells were incubated at 37 C with 1 nM [^{125}I]BSP or 100 nM [^{3}H]E1S with for different time periods. Data are expressed as percentage uptake of added radioactivity. Results are the means ± SEM of three experiments. a) P<0.05 for OATP1B1-Val^{174} vs. OATP1B1-Ala^{174} and vs. empty vector; b) P<0.05 for OATP1B1-Ala^{174} vs. empty vector.

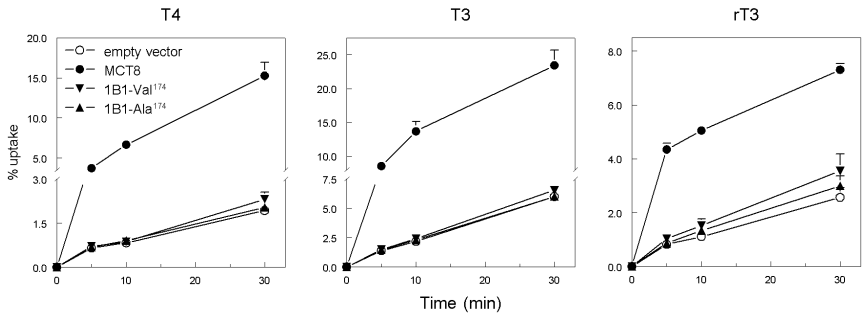

Figure 3: [^{125}I]T4, [^{125}I]T3 and [^{125}I]rT3 uptake by COS1 cells transfected with empty vector, OATP1B1-Val^{174}, OATP1B1-Ala^{174} or MCT8. Cells were co-transfected with CRYM, an intracellular thyroid hormone-binding protein. Cells were incubated for 5, 10 or 30 min at 37 C with 1 nM (2x10^{5} cpm) ^{125}I-labeled T4, T3 or rT3. Data are expressed as percentage uptake of added radioactivity. Results are the means ± SEM of three experiments. a) P<0.05 for OATP1B1-Val^{174} vs. OATP1B1-Ala^{174} and vs. empty vector; b) P<0.05 for OATP1B1-Ala^{174} vs. empty vector.

were wild type, 48 subjects were heterozygous and 3 subjects were homozygous for the OATP1B1-Val174Ala polymorphism, resulting in a minor-allele frequency of 17.7 %. In the Rotterdam Scan Study, the genotype distribution was quite similar with 714 wild type, 236 heterozygous and 27 homozygous subjects. In this cohort, the minor allele frequency was

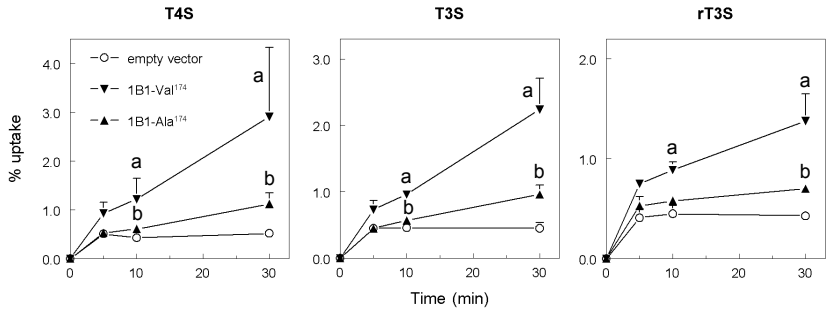

Figure 4: [^{125}I]T4S, [^{125}I]T3S and [^{125}I]rT3S uptake by COS1 cells transfected with empty vector, OATP1B1-Val^{174}, or OATP1B1-Ala^{174}. Cells were co-transfected with CRYM. Cells were incubated for 5, 10 or 30 min at 37 C with 1 nM [^{125}I]T4S or T3S. Data are expressed as percentage uptake of added radioactivity. Results are the means ± SEM of three experiments. a) P<0.05 for OATP1B1-Val^{174} vs. OATP1B1-Ala^{174} and vs. empty vector; b) P<0.05 for OATP1B1-Ala^{174} vs. empty vector.

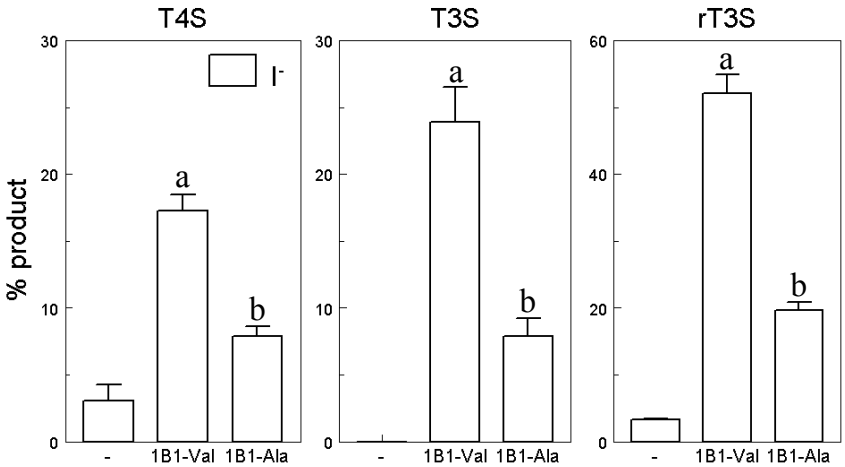

Figure 5: [^{125}I]T4S and [^{125}I]T3S metabolism by COS1 cells transfected with either rD1 alone or rD1 together with OATP1B1-Val^{174} or OATP1B1-Ala^{174}. Cells were incubated for 24 h at 37 C with 1 nM (1x10^{6} cpm) [^{125}I] T4S or T3S. Metabolism is shown as percentage of metabolites in the medium after 24 h incubation. Results are the means ± SEM of four experiments with T4S and three experiments with T3S and rT3S. a) P<0.05 for OATP1B1-Val^{174} vs. OATP1B1-Ala^{174} and vs. empty vector; b) P<0.05 for OATP1B1-Ala^{174} vs. empty vector.

14.8%, which is similar to previous studies in Caucasians (19). As only few homozygous subjects were identified, we combined heterozygotes and homozygotes into variant carriers.

In line with the *in vitro* findings, we found that bilirubin levels were 20 % higher in carriers of the OATP1B1-Ala^{174} allele compared to non-carriers (Table 2). Moreover, carriers had almost 40% higher E1S and 24% higher T4S than non-carriers.

Carriers of the Ala^{174} allele had higher rT3 levels in the cohort of healthy blood donors; however, this association failed to reach significance in the larger population of elderly Caucasians (Table 2). There was no association of the OATP1B1-Val174Ala polymorphism with serum TSH, FT4 and T3 levels (Table 2).

DISCUSSION

In this study we demonstrate that transfection of COS1 cells with OATP1B1 strongly induces uptake of BSP, E1S and the iodothyronine sulfates T4S, T3S and rT3S compared to mock transfected cells. Metabolism of iodothyronine sulfates by rD1 is greatly stimulated in the presence of OATP1B1. Moreover, OATP1B1-Val^{174} showed a 40% higher induction of transport and metabolism of these substrates than OATP1B1-Ala^{174}. These *in vitro* findings were supported by *in vivo* results showing that carriers of the OATP1B1-Ala^{174} allele had higher serum bilirubin, E1S and T4S levels.

Table 1: Baseline characteristics of the study populations

	Healthy blood donors	Rotterdam Scan Study
N	155	1012
Age (yrs)	46.42 ± 12.08	72.19 ± 7.38
Gender (M/F)	100 / 55	494 / 518
Bilirubin (µmol/L)	6 [5-7]	9 [7-11][a]
E1S (pmol/L)	-	207.6 [86.2-419.7][b]
TSH (mU/L)	1.19 [0.80-1.71]	1.15 [0.73-1.74]
FT4 (pmol/L)	15.1 ± 2.4	17.9 ± 3.0
T3 (nmol/L)	1.96 ± 0.24	2.00 ± 0.25
rT3 (nmol/L)	0.31 ± 0.08	0.34 ± 0.11
T4S (pmol/L)	16 [13-20]	-
OATP1B1- Val174Ala		
wild type	103 (66.5%)	714 (73.1%)
heterozygote	48 (31.6%)	236 (24.2%)
homozygote	3 (1.9%) (P=0.30)[c]	27 (2.8%) (P=0.17)[c]
Ala^{174} allele frequency	17.7%	14.8%

Data are shown as mean ± SD or as median [IQR].

a N =363, b N= 424, c P for deviation from Hardy-Weinberg equilibrium.

Table 2: Serum parameters by OATP1B1-Val174Ala genotype in healthy blood donors and in participants of the Rotterdam Scan Study

	Healthy blood donors			The Rotterdam Scan Study		
	OATP1B1-Val174Ala		P-value	OATP1B1-Val174Ala		P-value
	Wild type (103)	Carriers (49+3)		Wild type (714)	Carriers (236+27)	
Bilirubin[a]	6.0 ± 0.3	7.2 ± 0.5	0.02	9.3 ± 0.2[b]	10.2 ± 0.4[c]	0.04
E1S[a]	-	-		282.0 ± 16.2[d]	392.5 ± 27.9[e]	<0.001
TSH[a]	1.36 ± 0.07	1.23 ± 0.11	0.55	1.40 ± 0.04	1.36 ± 0.06	0.77
FT4	14.8 ± 0.2	15.6 ± 0.3	0.06	17.9 ± 0.1	18.1 ± 0.2	0.19
T3	1.96 ± 0.02	1.97 ± 0.03	0.64	2.01 ± 0.01	1.98 ± 0.02	0.15
rT3	0.30 ± 0.01	0.34 ± 0.01	0.006	0.34 ± 0.01	0.35 ± 0.01	0.08
T4S[a]	16.3 ± 0.6	20.1 ± 0.8	<0.001	-	-	

[a] Due to a non-normal distribution, E1S, bilirubin, T4S and TSH were logarithmically transformed in the analysis.
[b] N=268, [c] N=82+13, [d] N=317, [e] N=98+9

Sulfation is an important pathway in both thyroid hormone and estrogen metabolism. The physiological role of sulfated iodothyronines is not completely clear. The main function is probably the irreversible degradation of thyroid hormone by D1 (10). However, iodothyronine sulfates might also represent a reservoir of reversibly inactivated thyroid hormone when they are not degraded by D1, for instance in the fetus and during severe illness (30). Serum supply of estrogen sulfates and desulfation by estrogen sulfatase, on the other hand, plays an important role in the local formation of estrogens in different tissues (1, 14).

Although different OATPs are capable of sulfate conjugate transport, the major hepatic transporter for these conjugates has not been identified. In this study, we showed that transfection of cells with OATP1B1 induced a ~20-fold increased uptake rate of BSP. Moreover, OATP1B1-transfected cells showed considerable uptake of E1S, similar to previously published findings regarding uptake of these substrates (16). In the same way, transport of T4S, T3S and rT3S was greatly stimulated by OATP1B1 expression. Likewise, metabolism of these iodothyronine sulfates by D1 showed a marked increase when OATP1B1 was co-transfected. OATP1B1-Val^{174} showed a 40% higher induction of transport and metabolism of these substrates than OATP1B1-Ala^{174}. However, OATP1B1 did not induce T3 or T4 uptake, and we only found a minor increase in rT3 uptake. This is in contrast with findings published by Abe *et al.*, who showed T3 and T4 uptake in *Xenopus* oocytes injected with OATP1B1 cRNA (16). The discrepancy between their and our findings might be explained by differences in expression efficiency of the two systems, or the higher intracellular thyroid hormone-binding capacity of *Xenopus* oocytes compared to COS1 cells. Therefore, we co-transfected with CRYM, a high-affinity cytosolic thyroid hormone-binding protein (24), which has recently been shown to greatly augment net T3 and T4 uptake by inhibition of T3 and T4 efflux (31). Nevertheless, no significant uptake of T3 and T4 by OATP1B1 could be detected in the presence of CRYM either. Taken together, we conclude that in COS1 cells OATP1B1 has no T4 and T3 and only little rT3

transport activity, but does facilitate transport of iodothyronine sulfates and the previously used model substrates E1S and BSP. However, in a different transport assay system, OATP1B1 specific T4 and T3 transport has been demonstrated (16).

The physiological relevance of this transporter *in vivo* is underlined by the observation that carriers of the OATP1B1-Ala^{174} allele had higher bilirubin, E1S and T4S levels than non-carriers. This is in line with the *in vitro* data showing that uptake and metabolism of these substrates by the OATP1B1-Ala^{174} variant was less efficient compared to the OATP1B1-Val^{174} variant. Previously, Tirona and colleagues have also shown that the OATP1B1-Ala^{174} variant shows decreased uptake of E1S and estradiol-17β-glucuronide (19). A possible explanation for this altered transport function comes from a study performed by Kameyama and colleagues, who showed by immunocytochemistry that OATP1B1-Ala^{174} protein is not only localized at the plasma membrane, but also in the intracellular space, whereas OATP1B1-Val^{174} variant is only expressed at the plasma membrane (32). Therefore, the decreased activity of OATP1B1-Ala^{174} variant could be explained by a sorting error, resulting in decreased expression at the plasma membrane and thus decreased transport activity. In addition, kinetic analyses in transfected HEK293 cells have been performed showing that the differences in Km values were not apparent between OATP1B1-Val^{174} and OATP1B1-Ala^{174} variants. However, both the Vmax value and the intrinsic clearance were significantly decreased in cells expressing OATP1B1-Ala^{174} compared to OATP1B1-Val^{174}, probably due to the sorting error of the OATP1B1-Ala^{174} variant.

Breast cancer is one of the major causes of death in European and American women, occurring most frequently in postmenopausal women. Since estrogens have an important role in breast cancer, therapies aim to block interaction with the estrogen receptor by use of an anti-estrogen, or by inhibiting the conversion of androstenedione to estrone with an aromatase inhibitor (33, 34). Local formation of estrogens in breast tumors might be more important than circulating estrogens for growth and survival of estrogen-dependent breast cancer in post-menopausal women (35). Serum E1S is a major source of estrogens for breast tissue through local sulfatase activity (1). Since carriers of the OATP1B1-Val174Ala polymorphism have life-long higher serum E1S levels, the possible association of this polymorphism with risk and prognosis of breast cancer should be investigated.

In conclusion, OATP1B1 is an important factor in the hepatic transport and metabolism of bilirubin, E1S and iodothyronine sulfates. The OATP1B1-Ala^{174} variant allele displays decreased transport activity and thereby gives rise to higher serum bilirubin, E1S, and T4S levels in carriers of this polymorphism.

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Chapter 3

Thyroid hormone transport and metabolism by OATP1C1 and consequences of genetic variation

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ABSTRACT

OATP1C1 has been characterized as a specific thyroid hormone transporter. Based on its expression in capillaries in different brain regions, OATP1C1 is thought to play a key-role in transporting thyroid hormone across the blood-brain barrier. For this reason, we studied the specificity of iodothyronine transport by OATP1C1 in detail by analysis of thyroid hormone uptake in OATP1C1-transfected COS1 cells. Furthermore, we examined whether OATP1C1 is rate limiting in subsequent thyroid hormone metabolism in cells co-transfected with deiodinases. We also studied the effect of genetic variation in the OATP1C1 gene: polymorphisms were determined in 155 blood donors and 1192 Danish twins, and related to serum thyroid hormone levels. *In vitro* effects of the polymorphisms were analyzed in cells transfected with the variants.

Cells transfected with OATP1C1 showed increased transport of T4 and T4 sulfate (T4S), little transport of rT3 and no transport of T3 or T3S compared to mock transfected cells. Metabolism of T4, T4S and rT3 by co-transfected deiodinases was greatly augmented in the presence of OATP1C1. The OATP1C1-intron3C>T, Pro143Thr and C3035T polymorphisms were not consistently associated with TH levels, nor did they affect transport function *in vitro*.

In conclusion**,** OATP1C1 mediates transport of T4, T4S and rT3 and increases the access of these substrates to the intracellular active sites of the deiodinases. No effect of genetic variation on the function of OATP1C1 was observed.

INTRODUCTION

Organic anion transporting polypeptides (OATPs) are multi-specific sodium-independent transport proteins that are expressed in many tissues (1). In general, OATPs exhibit broad substrate specificity as they facilitate transport of a large variety of amphipathic organic compounds such as bile salts, lipid lowering drugs, but also thyroid hormones (TH) (2-6). In contrast to most OATPs, OATP1C1 shows high substrate specificity. It has been characterized as a high-affinity T4 and rT3 transporter with Km values in the nanomolar range, while other OATPs display Km values for TH transport far above the serum TH concentration (2, 3, 7). Based on the expression of OATP1C1 in capillaries in multiple brain regions, this protein is thought to play an important role in delivering serum TH to the brain (7, 8). Additional support for a significant role for OATP1C1 in TH transport in the brain comes from a study from Sugiyama and co-workers, in which they show that OATP1c1 is up-regulated in hypothyroid rats and down-regulated in hyperthyroid rats (8).

In this study, we examined the specificity of iodothyronine transport by OATP1C1 in transfected COS1 cells, and analyzed whether OATP1C1 is rate-limiting in subsequent iodothyronine metabolism in cells co-transfected with different deiodinases. In addition, we studied the effect of genetic variation in the OATP1C1 gene, as polymorphisms in different OATPs have been shown to alter the transport function of these proteins (9, 10). Identified polymorphisms were analyzed for association with serum thyroid parameters in two populations. In addition, the effect of the polymorphisms on the transport function of OATP1C1 was tested by analysis of uptake and metabolism of iodothyronines in cells transfected with OATP1C1 variants.

MATERIALS AND METHODS

Plasmids and construction of OATP1C1 variants

Human μ-crystallin (pSG5-hCRYM), rat type 1 deiodinase (pcDNA3-rD1), human type 2 deiodinase (pcDNA3-hD2-rD1-SECIS), and human type 3 deiodinase (pCIneo-hD3) plasmids were constructed as previously described (11). An OATP1C1 IMAGE clone (IMAGE 4801171) was purchased from the German Resource Center for Genome Research (www.rzpd.de) and subcloned into pcDNA3.1- (Invitrogen, Breda, The Netherlands) using *Not*I and *Acc65*I restriction sites.

The Pro143Thr polymorphism was introduced into the OATP1C1 cDNA using the Quick Change site-directed mutagenesis protocol (Stratagene, Amsterdam, The Netherlands) with forward primer 5'-CAAATATGAGAGATATTCTACTTCCTCCAATTCCACTCTCAGC-3' and the complementary reverse primer. The OATP1C1-3'UTR variants were constructed as follows: a PCR reaction was performed on pcDNA3.1-OATP1C1 with forward primer 5'-TGTGTG-

GAGCTGCAAAACTC -3' and reverse primer 5'-TGCAAAATG TCAACCAATTAGAAG-3', generating a PCR-product of 926 bp. A second PCR-reaction was performed on genomic DNA from a subject homozygous for the wild type or for the variant 3' UTR with forward primer 5'-TG-GGCACAGTGTCAATTCTC-3' and reverse primer 5'-CGTCGTGTA TAAGTAGGAAGTTGC-3', generating a product of 1107 bp. The final sequence of 1791 bp was generated by a PCR reaction of a 1:1 mixture of the above PCR products using the first forward primer and second reverse primer. This PCR-fragment was cloned into pCR-Blunt II TOPO (Invitrogen), excised with *BstE*II en *Acc65*I and shuttled into pcDNA3.1, yielding pcDNA3.1-OATP1C1-WT-3'UTR or VA-3'UTR.

Cell culture

COS1 cells were cultured in 6 or 24-well dishes (Corning, Schiphol, The Netherlands) with DMEM/F12 medium (Invitrogen), containing 9% heat-inactivated fetal bovine serum (Invitrogen) and 100 nM sodium selenite (Sigma).

Iodothyronine transport and metabolism

Materials: [^{125}I]NaI, [^{125}I]T4 and [^{125}I]T3 were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). [^{125}I]rT3 was purchased from Perkin Elmer (Boston, USA) and unlabeled T4, T3 and rT3 from Henning GmbH (Berlin, Germany). Unlabeled and ^{125}I-labeled T4 sulfate (T4S) and T3 sulfate (T3S) were synthesized as described previously (12). FuGENE6 transfection reagent was obtained from Roche Diagnostics (Indianapolis, IN).

Uptake studies: COS1 cells were cultured in 6-well plates and co-transfected with 500 ng empty pcDNA3.1 or pcDNA3.1-OATP1C1-Pro^{143}, Thr^{143}, WT-3'UTR or VA-3'UTR plus 500 ng pSG5-hCRYM, coding for a high-affinity cytosolic thyroid hormone-binding protein that prevents efflux of internalized iodothyronines (13). This protein binds not only T4 and T3, but also T4S and T3S (W.E. Visser, personal communication (14)). However, the affinity of hCRYM for rT3 and rT3S is much lower compared to the other iodothyronines.

After 24 h culturing, cells were washed with incubation medium (DMEM-F12 with 0.1% BSA), and incubated for 15, 30 or 60 min at 37 C with 1 nM (2x10^5 cpm) [^{125}I]T4, [^{125}I]T3, [^{125}I]rT3, [^{125}I]T4S or [^{125}I]T3S in 1.5 ml incubation medium. After incubation, cells were harvested and analyzed as described previously (11). For determination of kinetic parameters, cells were incubated with 0.01-1 µM [^{125}I]T4 or 0.1-5 µM [^{125}I]T4S. Data were corrected for background uptake in cells transfected with empty vector instead of OATP1C1.

Metabolism studies: COS1 cells were cultured in 24-well dishes and transfected with 100 ng empty vector plus 100 ng rD1, hD2, or hD3 plasmid or with 100 ng deiodinase plus 100 ng OATP1C1 plasmid. After 24 h culturing, cells were incubated for 24 h at 37 C with 1 nM (1 x 10^6 cpm) ^{125}I-labeled T4, rT3 or T4S in 0.5 ml incubation medium. After incubation, medium was harvested and analyzed by HPLC as described previously (11). Production of the metabolites

is presented as percentage of total radioactivity in the medium, which represents >90% of added radioactivity.

Identification of polymorphisms and haplotypes in the OATP1C1 gene

The structure of the OATP1C1 gene was determined using the NCBI gene database. The polymorphisms in the region were obtained from databases of the International Hapmap Project (http://www.hapmap.org) (15) and the National Center for Biotechnology Information (NCBI) dbSNP (http://www.ncbi.nlm.nih.gov). In addition, DNA of 25 randomly selected Caucasian subjects was used for sequence analysis of the complete coding region, including intron/exon boundaries, of the OATP1C1 gene to verify polymorphisms found in different databases and to identify novel polymorphisms. Primers used for amplification and sequencing are available on request.

The haploblock structure of the gene was determined using Haploview v3.32 (16) according to the method of Gabriel *et al*. (17). A haploblock is a set of statistically associated polymorphisms, and Haploview is a program designed to analyze and visualize patterns of linkage disequilibrium, i.e. non-random associations between polymorphisms.

Study populations

We analyzed a population of 154 healthy blood donors from the Sanquin Blood Bank South West region (Rotterdam, The Netherlands) (18). Informed consent was given by all donors. Serum TSH, T4, T3 and free T4 (FT4) were determined as described previously (18). T4S was determined by a specific RIA (19).

The second population consisted of participants of a nation-wide project (GEMINAKAR) investigating the relative influence of genetic and environmental factors on a variety of traits among Danish twins. Based on a questionnaire survey concerning physical health and health related behavior performed in 1994, a representative sample of twin pairs was recruited from the population-based Danish Twin Registry (20). A detailed description of the ascertainment procedure can be found elsewhere (21-23). In the GEMINAKAR study 1512 individuals (756 twin pairs) were examined. Blood samples for thyroid measurements and genotype information were available in 1266 individuals, distributed in 554 monozygotic (MZ), 474 same sex dizygotic (DZ) and 238 opposite sex (OS) twin individuals. In the MZ twin pairs, in which only one of the twins was genotyped, we assumed identical genotypes. Twin pairs in which one or both twins had self-reported thyroid disease (22 twin pairs) or biochemical thyroid disease (15 twin pairs) were excluded, leaving 1192 (524 MZ, 442 DZ and 226 OS twin individuals). Serum thyroid parameters were determined as described previously (21, 24). Written informed consent was obtained from all participants and the study was approved by all regional Danish Scientific-Ethical Committees (case file 97/25 PMC).

In the case of significant associations the use of twins would allow us to assess the contribution of these polymorphisms to the trait variation and the genetic variance.

Genotyping

The polymorphisms OATP1C1-intron3 C>T (rs10770704), OATP1C1-Pro143Thr (rs36010656) and OATP1C1-C3035T (rs10444412) were determined by 5'fluorogenic Taqman assays. Reactions were performed in 384-wells format on ABI9700 PCR machines with endpoint reading on the ABI 7900HT Taqman machine (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) (25).

Statistical analysis

Data were analyzed using SPSS 10.0.7 for Windows (SPSS, Inc., Chicago, IL) and STATA statistical software (STATA Corporation, College Station, TX). P values are two-sided throughout and were considered significant if $P < 0.05$.

For the *in vitro* experiments, unpaired Student's t-tests were used to test whether iodothyronine uptake and metabolism induced by wild-type OATP1C1 was significantly different from cells transfected with variant OATP1C1 or empty vector.

The polymorphisms were analyzed for deviation from Hardy-Weinberg equilibrium proportions using a Chi-square test. In the cohort of blood donors, the distribution of serum TSH was skewed and therefore transformed by the natural logarithm. Differences between genotypes were adjusted for age and gender and tested by analysis of covariance (ANCOVA). In case of an allele dose effect for either one of the polymorphisms, a linear regression analysis was performed to quantify the association.

In the Danish twins, associations between the OATP1C1-C3035T polymorphism and serum thyroid parameters were assessed using regression analysis. The statistical inference measures were computed using a technique (the cluster option in STATA) that takes the dependency of twin data into account. The genotype information was incorporated in the regression analyses by coding the noncarriers as 0, heterozygous carriers as 1 and homozygote carriers as 2. Due to non-normal distribution of most of the serum parameters were transformed by the natural logarithm, and adjustment for age and gender was performed.

RESULTS

Transport of (sulfated) iodothyronines by OATP1C1

Incubation of OATP1C1-transfected COS1 cells with [^{125}I]T4 resulted in a significant stimulation of the uptake of this iodothyronine over cells transfected with empty vector (Fig. 1A). In addition, cellular uptake of [^{125}I]rT3 was induced in cells transfected with OATP1C1 compared to mock transfected cells, however this failed to reach significance (Fig. 1A). The low affinity of rT3 for CRYM may at least in part explain why its uptake was lower than that of T4 and not linear with incubation time (13). Cellular uptake of [^{125}I]T3 was not stimulated after transfec-

tion with OATP1C1 (Fig. 1A). The high basal [^{125}I]T3 uptake in cells transfected with empty vector and CRYM is probably due to T3 uptake by endogenous transporters in COS1 cells.

In addition to T4, rT3 and T3, OATP1C1-transfected cells were tested for uptake of sulfated iodothyronines T4S and T3S. OATP1C1 did not facilitate uptake of [^{125}I]T3S. However, [^{125}I] T4S uptake was increased in OATP1C1-transfected cells compared to mock transfected cells (Fig. 1A). In addition, the saturation kinetics of T4 and T4S uptake by OATP1C1 were studied by incubation of OATP1C1-transfected COS1 cells during 30 min with 0.01-1 µM T4 or 0.1-5 µM T4S. The results are presented in Figure 1B, showing that both T4 and T4S uptake were saturable with apparent Km values of 0.12 µM for T4 and 2.6 µM for T4S. Vmax values were similar for T4 and T4S (0.25 vs. 0.40 nmol/min).

Iodothyronine metabolism in cells co-transfected with OATP1C1 and deiodinases

Incubation of cells transfected with D2 or D3 led to some conversion of T4. However, this was markedly and significantly increased, when cells were co-transfected with OATP1C1 (Fig. 2).

Based on the transport assays, it is not entirely clear whether OATP1C1 transports [^{125}I] rT3 in a significant manner. However, metabolism of rT3 by either D1 or D2 was increased 2-fold in cells co-transfected with OATP1C1 (Fig. 2). In addition, T4S metabolism by D1 was increased 10-fold in cells co-transfected with OATP1C1 (Fig 2).

Identification of polymorphisms in the OATP1C1 gene

Sequence analyses of 25 Caucasian subjects revealed 13 polymorphisms, among which one novel sequence variation in the OATP1C1 gene (Fig. 3). For this study, we preferentially select-

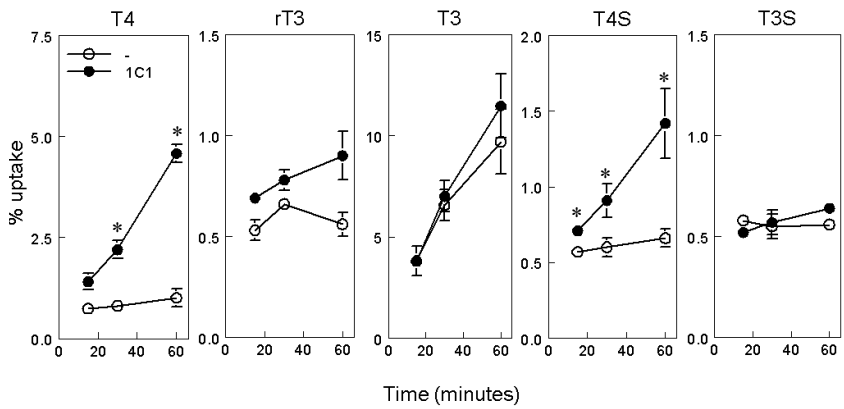

Figure 1A: [^{125}I]T4, [^{125}I]rT3, [^{125}I]T3, [^{125}I]T4S and [^{125}I]T3S uptake by COS1 cells transfected with empty vector or OATP1C1. Cells were co-transfected with CRYM, an intracellular thyroid hormone-binding protein. Cells were incubated for 15, 30 or 60 min at 37 C with 1 nM ($2x10^5$ cpm) ^{125}I-labeled T4, rT3, T3, T4S or T3S. Data are expressed as percentage uptake of added radioactivity. Results are the means ± SEM of at least two experiments. * P<0.05, ** P<0.01 of cells transfected with OATP1C1 versus mock transfected cells.

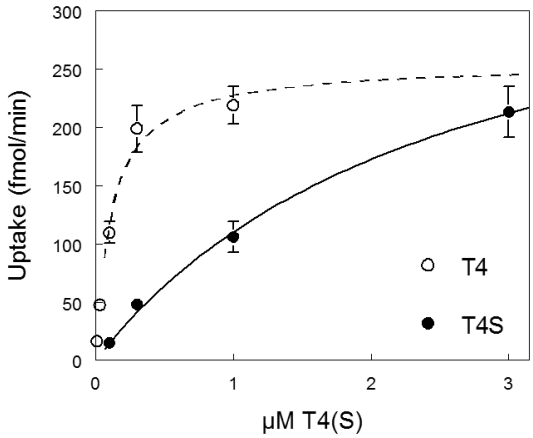

Figure 1B: Ligand concentration-dependent uptake of T4 and T4S by OATP1C1-transfected COS1 cells. Cells were incubated for 30 min with 0.01-1 µM [^{125}I]T4 or 0.1-5 µM [^{125}I]T4S. Data were corrected for background uptake in cells transfected with empty vector instead of OATP1C1.Curve-fitting was performed using the Michaelis-Menten equation $v = V_{max}/(1+K_m/S)$. Results are the means ± SEM of 4-6 observations.

ed non-synonymous polymorphisms or polymorphisms in the 3'UTR. One non-synonymous polymorphism, OATP1C1-Pro143Thr, and 4 polymorphisms in the 3'UTR were selected. By analyzing the linkage disequilibrium block structure across the gene from the Hapmap data (Phase II release 22) using the Haploview program (16), the identified polymorphisms in the 3'UTR were found to be part of a haploblock spanning from exon 10 to the end of the 3'UTR in exon 15. Therefore, the C3035T polymorphism was genotyped as a tagging polymorphism for the other polymorphisms in this haploblock. In an attempt to cover the remainder of the gene, a C>T polymorphism in intron 3 (rs10770704) was genotyped as a tagging polymorphism for the first haploblock encompassing the first 4 exons (Fig. 3).

OATP1C1 polymorphisms in healthy blood donors

The baseline characteristics of the population of blood donors are shown in Table 1. One subject failed genotyping for the OATP1C1-intron3C>T polymorphism. All polymorphisms were in Hardy-Weinberg equilibrium.

The OATP1C1-intron3C>T polymorphism was not associated with serum thyroid parameters (data not shown). For the OATP1C1-Pro143Thr polymorphism, all serum thyroid parameters were similar between carriers and non-carriers, except for rT3, which was higher in carriers of the OATP1C1-Thr^{143} allele (Table 2). The OATP1C1-C3035T polymorphism was in a dose-dependent manner associated with a higher serum FT4 and a higher rT3 (Table 2).

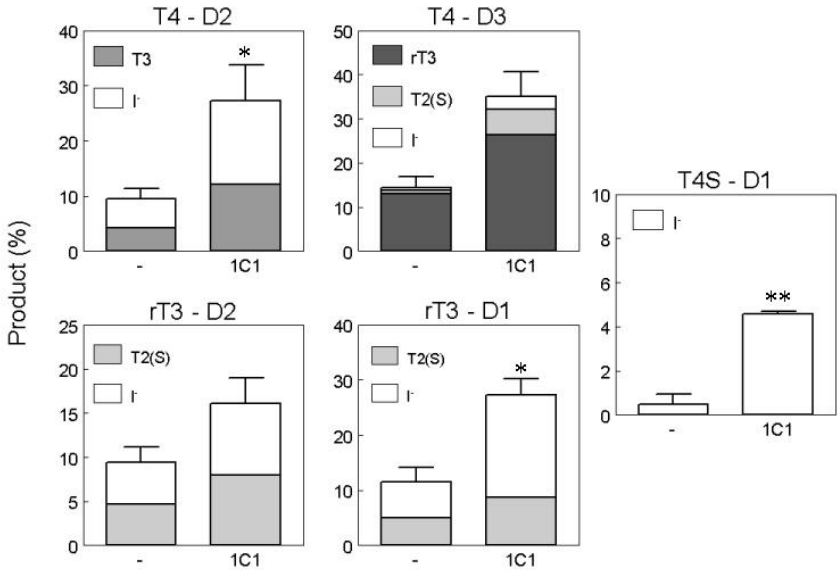

Figure 2: [^{125}I]T4, [^{125}I]rT3 and [^{125}I]T4S metabolism by COS1 cells transfected with either D1, D2 or D3 alone or together with OATP1C1. Cells were incubated for 24 h at 37 C with 1 nM (1x10^6 cpm) ^{125}I-labeled T4, rT3 or T4S. Metabolism is shown as percentage of metabolites in the medium after 24 h incubation. Results are the means ± SEM of at least three experiments. * P<0.05, ** P<0.01 of cells co-transfected with OATP1C1 and deiodinase versus cells co-transfected with empty vector and deiodinase.

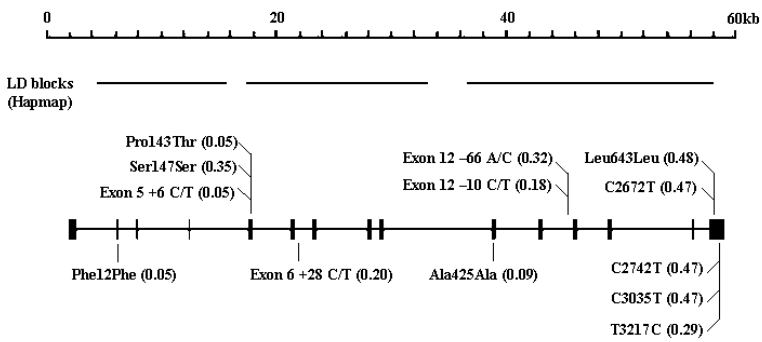

Figure 3: Schematic overview of the *OATP1C1* gene (Chr. 12p12.2). Nucleotide numbers are based on Genbank accession number NM 017435.2. The vertical black boxes represent the 15 exons of the OATP1C1 gene, and the vertical lines represent the polymorphisms found by direct sequencing of 25 Caucasian blood donors. The numbers between parentheses represent the minor allele frequencies of the different polymorphisms. The horizontal lines above the gene structure are a schematic representation of the 3 linkage disequilibrium (LD) blocks according to Hapmap data (Hapmap release II, phase 21).

OATP1C1 polymorphisms in healthy Danish Twins

Based on the results of the association analysis of the OATP1C1 polymorphisms in the cohort of blood donors, we only genotyped the OATP1C1-C3035T polymorphism in the population of Danish twins. The baseline characteristics of this population are shown in Table 1. The allele frequency of the OATP1C1-C3035T polymorphism was 41.7%, which is almost 6% lower than the frequency observed in the population of healthy blood donors.

In contrast to our findings in the blood donors, no significant differences in serum thyroid parameters were observed between subjects carrying 0, 1 or 2 copies of this polymorphism (Table 3). Therefore, we refrained from assessing the contribution of this polymorphism to the variation in serum thyroid parameters and the genetic variance.

Effect of OATP1C1 polymorphisms in vitro

The *in vitro* effect of OATP1C1-Pro143Thr and the polymorphisms in the 3'UTR was tested by transfecting COS1 cells with these variants and analyzing them for differences in T4 and rT3 uptake and metabolism from wild-type OATP1C1. For OATP1C1-Pro143Thr no significant

Table 1: Baseline characteristics of the study populations

		Dutch blood donors	Danish twins
N		154	1192
Age (yrs)		46.32 ± 12.06	36.67 ± 10.72
Gender (M/F)		99 / 55	601/591
TSH (mU/L)		1.19 [0.80-1.71]	1.56 [1.14-2.17]
FT4 (pmol/L)		15.08 ± 2.40	12.82 ± 1.63
T4 (nmol/L)		87 ± 16	111 [100 - 127]
T3 (nmol/L)		1.96 ± 0.24	1.84 [1.66 - 2.09]
rT3 (nmol/L)		0.31 ± 0.08	0.35 [0.29 - 0.43]
T4S		16 [13-20]	-
Intron3C>T	wild-type (%)	50 (32.7)	-
	heterozygote (%)	70 (45.8)	
	homozygote (%)	33 (21.6)	
	allele frequency (%)	44.4	
	HWE-p-value	0.36	
Pro143Thr	wild-type (%)	140 (90.9)	-
	heterozygote (%)	14 (9.1)	
	homozygote (%)	0 (0.0)	
	allele frequency (%)	4.5	
	HWE-p-value	0.55	
C3035T	wild-type (%)	45 (29.2)	398 (33.4)
	heterozygote (%)	73 (47.4)	594 (49.8)
	homozygote (%)	36 (23.4)	200 (16.8)
	allele frequency (%)	47.1	41.7
	HWE-p-value	0.55	0.39

Data are shown as mean ± SD or as median [IQR]. HWE-p-value represents the p-value for deviation from Hardy-Weinberg equilibrium.

Table 2: Serum thyroid parameters by OATP1C1 genotypes in a population of Dutch blood donors

	OATP1C1-Pro143Thr		P[a]	OATP1C1-C3035T			P[b]
	wild-types (140)	carriers (14)		wild-types (45)	heterozygotes (73)	homozygotes (36)	
TSH	1.31 ± 0.06	1.33 ±0.11	0.69	1.29 ± 0.11	1.29 ± 0.09	1.39 ± 0.13	0.36
T4	87.47± 1.30	91.91 ± 4.11	0.30	86.51 ± 2.31	87.82 ± 1.81	89.68 ± 2.58	0.36
FT4	15.01 ± 0.20	15.71 ± 0.64	0.30	14.38 ± 0.35	15.27 ± 0.28	15.56 ± 0.39	0.02
T3	1.96 ± 0.02	2.02 ± 0.05	0.33	2.01 ± 0.04	1.96 ± 0.03	1.91 ± 0.04	0.06
rT3	0.31 ± 0.01	0.36 ± 0.02	0.03	0.29 ± 0.01	0.31 ± 0.01	0.34 ± 0.01	0.008
T4S	17.5 ± 0.5	18.5 ± 1.6	0.41	17.0 ± 0.9	17.3 ± 0.7	18.8 ± 1.0	0.16

Data are shown as mean ± SE.

[a] P-value for ANOVA, adjusted for age and gender

[b] P-value for linear regression, adjusted for age and gender

Table 3: Serum thyroid parameters by OATP1C1-C3035T genotype in a population of Danish twins

	OATP1C1-C3035T			P[a]
	wild-types (398)	heterozygotes (594)	homozygotes (200)	
TSH	1.73 ± 0.89	1.77 ± 0.95	1.76 ± 0.94	0.54
T4	114.4 ± 25.3	117.1 ± 25.4	117.5 ± 25.1	0.06
FT4	12.73 ± 1.59	12.87 ± 1.65	12.84 ± 1.67	0.37
T3	1.89 ± 0.37	1.92 ± 0.41	1.91 ± 0.36	0.52
rT3	0.36 ± 0.11	0.37 ± 0.11	0.36 ± 0.10	0.85

Data are shown as mean ± SD

[a] P-value for linear regression on the transformed values, adjusted for age and gender

differences in uptake and metabolism of T4 compared to wild-type OATP1C1 were observed (Fig. 4A, B). In addition, no difference in T4 uptake and T4 metabolism was observed between cells transfected with OATP1C1- WT-3'UTR or VA-3'UTR (Fig. 4A,B). Neither could we detect any differences in rT3 uptake and metabolism between the different OATP1C1 variants (data not shown).

DISCUSSION

In this study we demonstrated that T4, T4S and to some extent rT3 uptake was induced in cells transfected with OATP1C1 compared to mock transfected cells. In addition, metabolism of T4, T4S and rT3 by transfected deiodinases was markedly stimulated in the presence of OATP1C1. Although, the OATP1C1-Pro143Thr and OATP1C1-C3035T polymorphisms were associated with serum thyroid parameters in 155 blood donors, we could not replicate these findings in a much larger cohort of Danish twins. Nor did we observe any difference in uptake and metabolism of T4 and rT3 between these variants and wild-type OATP1C1.

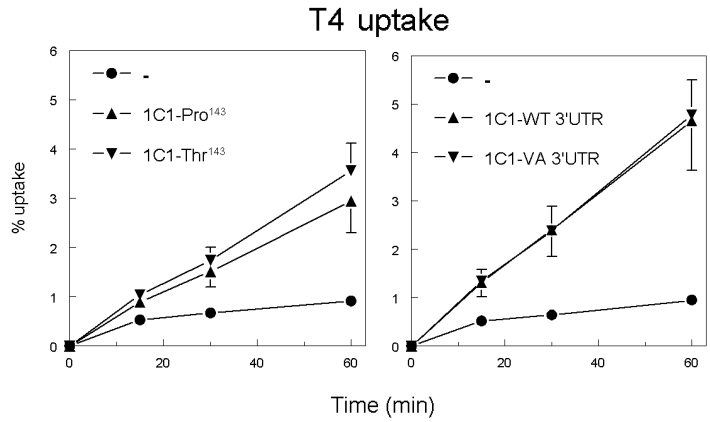

Figure 4A: Uptake of [^{125}I]T4 by COS1 cells transfected with empty vector or one of the OATP1C1 variants. Cells were co-transfected with CRYM. Cells were incubated for 15, 30 or 60 min at 37 C with 1 nM (2x10^5 cpm) [^{125}I]T4. Data are expressed as percentage uptake of added radioactivity. Results are the means ± SEM of at least three experiments. * P<0.05, ** P<0.01 of cells transfected with wild-type OATP1C1 versus cells transfected with variant OATP1C1

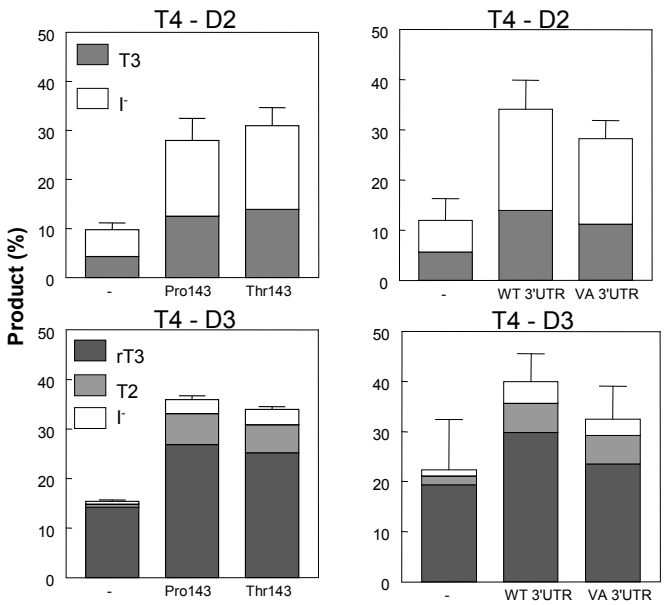

Figure 4B: Metabolism of [^{125}I]T4 by COS1 cells transfected with either D2 or D3 alone or together with one of the OATP1C1 variants. Cells were incubated for 24 h at 37 C with 1 nM (1x10^6 cpm) ^{125}I-labeled T4. Metabolism is shown as percentage of metabolites in the medium after 24 h incubation. Results are the means ± SEM of at least three experiments. * P<0.05, ** P<0.01 of cells co-transfected with wild-type OATP1C1 and deiodinase versus cells co-transfected with variant OATP1C1 and deiodinase

Based on its lipophilic structure, it was assumed that thyroid hormone enters the cell through passive diffusion (26). However, with the discovery of MCT8 as a specific thyroid hormone transporter and the fact that mutations in this transporter lead to the Allan-Herndon-Dudley syndrome (OMIM 300523), the interest in this area of research has greatly increased (27-29). In this paper we focused on OATP1C1, which was first described by Pizzagalli and colleagues as a specific thyroid hormone transporter (7). OATP1C1 is expressed in multiple brain regions and in the testis. In line with findings by Pizzagalli *et al.*, we found a clear induction of T4 uptake and to some extent also of rT3 uptake by cells transfected with OATP1C1. Furthermore, metabolism of T4 and rT3 by D1, D2 or D3 was markedly increased if OATP1C1 was co-transfected. This demonstrates that OATP1C1 expression is rate limiting in iodothyronine metabolism by the deiodinases. It thus supports the hypothesis that OATP1C1 indeed increases the intracellular availability of these iodothyronines, since the deiodinases are membrane proteins with their active sites located intracellularly (30).

It should be noted that co-transfection with deiodinases was done to monitor the increase in intracellular iodothyronine concentration by OATP1C1 rather than to mimic particular tissue cells. OATP1C1 is probably only co-expressed with D2 *in vivo*. Both OATP1C1 and D2 are expressed in tanycytes that line the third ventricle (31). Conversion of T4 to T3 in these cells plays an important role in the negative feedback of thyroid hormone at the hypothalamus. OATP1C1 and D2 are also co-expressed in the testis, which may also facilitate local T3 formation in this tissue (32).

We further extended the findings from Pizzagalli *et al.*, by showing that OATP1C1 also facilitates transport and subsequent metabolism of T4S, but not of T3S. The serum concentrations of T4S and T3S are low under normal conditions as sulfation of thyroid hormone accelerates its degradation by D1 (19, 30, 33-36). However, serum iodothyronine sulfate levels are high in preterm infants and during critical illness (33, 35, 37). Under these conditions, the sulfates might serve as a reservoir of inactive thyroid hormone, from which active thyroid hormone can be recruited when necessary. It is, therefore, interesting that T4S is a ligand for OATP1C1-mediated transport. Since Oatp1c1 is localized both on the luminal and abluminal membrane of brain capillaries in rats and mice, this protein could play a role in uptake as well as efflux of T4S across the blood-brain barrier (8). During embryonic development, uptake of T4S across the blood-brain barrier by OATP1C1, may serve as source of T4 for the brain after local hydrolysis by sulfatases. OATP1C1 might, however, also serve as an export pump for T4S. Several sulfotransferases are expressed in different brain regions (38, 39). Among these are the most potent iodothyronine sulfotransferases SULT1A1 and SULT1E1 (40, 41). In addition to the action of the deiodinases, these enzymes might tightly regulate T4 levels in different brain regions.

The Michaelis-Menten transport kinetics of T4 and T4S transport by OATP1C1 were determined. Both were saturable with apparent Km values of 0.12 μM for T4 and 2.6 μM for T4S. It is important to realize that these are approximate values as in addition to OATP1C1 binding of

T4 and T4S to BSA and CRYM are also saturable processes. Nevertheless, our Km value for T4 is in good agreement with that previously reported by Pizzagalli *et al.* (90 nM) (7).

Based on the function of OATP1C1, polymorphisms in the OATP1C1 gene might be associated with T4, T4S and rT3 levels (7). As the serum FT4 concentration (~15 pM) is orders of magnitude lower than the apparent Km value (~100 nM), the rate of T4 transport by OATP1C1 *in vivo* is linearly dependent on the Vmax/Km ratio. Polymorphisms that change the Vmax or Km value would directly affect tissue T4 uptake by OATP1C1.

Carriers of the OATP1C1-Pro143Thr polymorphism indeed had higher rT3 levels than non-carriers in a population of healthy blood donors. Furthermore, the OATP1C1-C3035T polymorphism was in a dose-dependent manner associated with higher serum FT4 and serum rT3 in this same cohort. In contrast to our findings in the Dutch blood donors, no significant differences in serum thyroid parameters were observed between carriers of 0, 1, or 2 copies of the OATP1C1-C3035T polymorphism in the Danish twins. In addition, in a third population of approximately 1000 Caucasian subjects, the OATP1C1-C3035T polymorphism was not significantly associated with serum thyroid hormone levels (unpublished data by W.M. van der Deure, R.P. Peeters and T.J. Visser).

Furthermore, uptake and metabolism of T4 and rT3 by COS1 cells transfected with the OATP1C1 variants was similar to cells transfected with cDNA coding for the wild-type form of the OATP1C1 protein. It is, therefore, likely that our initial findings in the blood donors were chance findings, which might be caused by the small sample size of this cohort (42). However, differences in age and environmental factors such as iodine intake between the two populations might also in part explain the inconsistent findings.

Although we did not observe an effect of polymorphisms in the OATP1C1 gene on serum thyroid parameters, this might not rule out local effects of genetic variation. For instance, the Thr92Ala polymorphism in D2 is not associated with serum thyroid parameters or differences in iodothyronine metabolism in transiently transfected COS1 cells (18), but has been associated with insulin resistance in different populations (43, 44). Therefore, we cannot exclude that polymorphisms in the OATP1C1 gene could be associated with brain-related phenotypes, such as depression or cognition.

In conclusion, our findings indicate that OATP1C1 mediates plasma membrane transport of T4, T4S and rT3 and is rate limiting in iodothyronine metabolism by the deiodinases. Polymorphisms in the OATP1C1 gene are not associated with serum thyroid parameters, nor do they alter the transport function of OATP1C1.

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Chapter 4

Polymorphisms in the brain-specific thyroid hormone transporter OATP1C1 are associated with fatigue and depression in hypothyroid patients

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ABSTRACT

Introduction: Some hypothyroid patients continue to have significant impairments in psychological well-being, despite adequate treatment with levothyroxine (LT4). T4 transport across the blood-brain barrier is one of the crucial processes for thyroid hormone action in the brain. OATP1C1, a thyroid hormone transporter expressed at the blood-brain barrier, is thought to play a key role in delivering serum T4 to the brain.

Objective: To examine whether polymorphisms in OATP1C1 are determinants of well-being, neurocognitive functioning and preference for replacement therapy with a combination of LT4 and liothyronine (LT3).

Design and participants: We studied 141 patients with primary autoimmune hypothyroidism, adequately treated with LT4 monotherapy and participating in a randomized clinical trial comparing LT4 therapy with LT4/LT3 combination therapy.

Outcome measurements: Different questionnaires on well-being and neurocognitive tests were performed at baseline. Serum thyroid parameters, OATP1C1-intron3C>T, OATP1C1-Pro143Thr and OATP1C1-C3035T polymorphisms were determined.

Results: Allele frequencies of the OATP1C1 polymorphisms in patients with primary hypothyroidism were similar to those of healthy controls. Both the OATP1C1-intron3C>T and the OATP1C1-C3035T polymorphism, but not the OATP1C1-Pro143Thr polymorphism, were associated with symptoms of fatigue and depression. OATP1C1 polymorphisms were not associated with measures of neurocognitive functioning or preference for combined LT4/LT3 therapy.

Conclusions: OATP1C1 polymorphisms are associated with fatigue and depression, but do not explain differences in neurocognitive functioning or appreciation of LT4/LT3 combination therapy. Future studies are needed to confirm these findings.

INTRODUCTION

Adequate levels of thyroid hormone are critical for normal brain function. This is clearly demonstrated by the increased prevalence of mood disorders in patients with overt thyroid disease (1). Some hypothyroid patients remain to have significant impairments in psychological well-being (2) and neurocognitive functioning (3), despite adequate treatment with levothyroxine (LT4). This might be because standard replacement therapy is not sufficient to restore euthyroidism in all tissues (4). One randomized clinical trial of combined LT4 and liothyronine (LT3) therapy versus LT4 monotherapy in hypothyroid patients showed that combination therapy improved mood, well-being and cognitive functioning in comparison with treatment with LT4 alone (5). However, several more recent trials, including our own, have not replicated this finding (6-10).

Hypothyroid patients treated with LT4 probably derive T3 exclusively from exogenously administered LT4, as these patients lack normal T3 production by the thyroid gland. Therefore, proper function of key-proteins involved in transport and metabolism of T4 are of utmost importance. By inference, polymorphisms in the genes encoding these proteins might well be determinants of well-being in this group of patients. For instance the type 2 deiodinase (D2), which regulates the conversion of T4 to T3 in the brain, might be essential (11). However, we have recently shown that polymorphisms in D2 are not associated with differences in well-being or neurocognitive functioning in patients adequately treated for hypothyroidism (12).

Since all T4 in the brain is derived from serum, thyroid hormone transport across the blood-brain barrier is a crucial process for thyroid hormone action in the brain. Several proteins capable of thyroid hormone transport have been identified, such as members of the monocarboxylate (MCT) and organic anion transporting polypeptide (OATP) families (13). MCT8 has been characterized as an active thyroid hormone transporter and mutations in this transporter lead to the Allan-Herndon-Dudley syndrome (OMIM 300523) (14-16). OATP1C1, located on chromosome 12p12, is capable of T4 and rT3 transport (17). Based on its expression at the blood-brain barrier, OATP1C1 is thought to play a key role in delivering serum T4 to the brain (17, 18). Additional support for a significant role for OATP1C1 in thyroid hormone transport in the brain comes from a study from Sugiyama and co-workers, in which they show that OATP1c1 is up-regulated in hypothyroid rats and down-regulated in hyperthyroid rats (18). Hence, subtle changes in transporter function may influence transport of thyroid hormone into the brain and thus alter local T4 concentrations in the brain.

Recently, we identified several polymorphisms in the OATP1C1 gene (Fig. 1) (19), of which OATP1C1-intron3C>T, OATP1C1-Pro143Thr and OATP1C1-C305T were genotyped in this study. OATP1C1-intron3C>T is a tagging polymorphism for a haploblock encompassing the first four exons of the OATP1C1 gene, whereas OATP1C1-C3035T captures most of the genetic variation in a haploblock, spanning from exon 10 until the end of the OATP1C1 gene (Fig. 1)

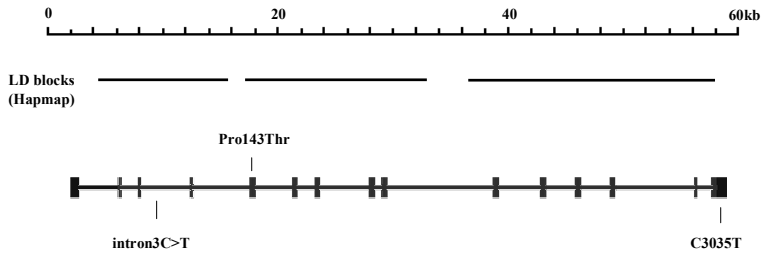

Figure 1: Schematic overview of the OATP1C1 gene on chromosome 12p12.2 (NM 017435) showing the polymorphisms genotyped in this population together with haploblocks based on the Hapmap project (Hapmap release II, phase 21).

(19). To date, OATP1C1-Pro143Thr is the only non-synonymous polymorphism identified in the OATP1C1 gene. We analyzed whether these polymorphisms are determinants of well-being and neurocognitive functioning in hypothyroid patients on adequate LT4 therapy. Secondly, we investigated whether OATP1C1 genotypes are associated with a preference for LT4 or LT4 and LT3 replacement therapy.

PATIENTS AND METHODS

Patients

Patients, between 18 and 70 years of age, who had been on an adequate dose of LT4 replacement therapy as reflected by normalized serum TSH levels for primary autoimmune hypothyroidism for at least 6 months, were recruited from 13 general practices in the cities of Amsterdam and Almere. The patients participated in a randomized controlled trial investigating possible superiority of therapy with a LT4/LT3 combination in a ratio of 5:1 or 10:1 over therapy with LT4 alone (9). For more detailed information on the patients and the study design, please refer to (9). The protocol was approved by the institutional review board of the Academic Medical Center of the University of Amsterdam. Informed consent was obtained from all participants.

Questionnaires and neurocognitive tests

Well-being of patients was measured by means of self-report questionnaires and neurocognitive functioning by tests administered by a trained psychometrician under supervision of a clinical neuropsychologist, all performed before the study medication of the randomized trial was supplied. Subjective appreciation of the study medication, compared with the usual LT4 medication from before the trial, was rated at every visit and at endpoint after 15 weeks. The set of questionnaires included the 32-item Profile of Moods States Dutch shortened ver-

sion (POMS), designed to monitor changes in mood states (20); the original Dutch version of the Multidimensional Fatigue Inventory (MFI-20), consisting of 20 questions and designed to measure (changes in) fatigue (21); the mental health and vitality subscales of the Rand 36-item health survey (Rand-36) (22); and the Dutch version of the Symptom Checklist (SCL-90), a 90 item self-report scale containing eight subscales measuring multidimensional psychopathology (23).

Measurement of neurocognitive functioning included tests of attention and working memory (Digit Span subtest of the Wechsler Adult Intelligence Scale) (24); learning and memory (Dutch version of the Story Recall from the Rivermead Behavioral Memory Test (RBMT)) (25, 26), and the Dutch adaptation of the California Verbal Learning Test (CVLT) (27, 28); psychomotor speed (Dutch adaptation of the Digit Symbol subtest of the Wechsler Adult Intelligence Scale-III) (24, 29); speed of memory processing (Memory Comparison Task (MCT), computer version (30), and paper-and-pencil version (31); and attention (Dutch adaptation of the Paced Auditory Serial Attention Task) (32, 33).

Serum analyses

All blood samples were collected in the morning before medication was ingested, approximately 24 h after the LT4 dose. Serum TSH and FT4 were measured by time-resolved fluoroimmunoassay (Wallac Oy, Turku, Finland). Serum T4 and T3 were measured by in-house RIA methods (34).

Genotyping

DNA was isolated from peripheral leucocytes by standard procedures. Three polymorphisms in the OATP1C1 gene (OATP1C1-intron3C>T (rs10770704), OATP1C1-Pro143Thr (rs36010656) and OATP1C1-C3035T (rs10444412)) were determined by 5'fluorogenic Taqman assays. Reactions were performed in 384-wells format on ABI9700 2x384 well PCR machines with endpoint reading on the ABI 7900HT Taqman machine (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) (35).

DNA was available of 141 patients. The genotyping success rate for OATP1C1-Pro143Thr and OATP1C1-C3035T was 100%. For OATP1C1-intron3C>T one sample failed genotyping.

Statistical analyses

Data were analyzed using SPSS 10.0.7 for Windows (SPSS, Inc., Chicago, IL). For the three polymorphisms, deviation from Hardy-Weinberg equilibrium proportions was analyzed using a Chi-square test. Differences between genotypes in serum thyroid parameters, scores of well-being and neurocognitive functioning were adjusted for age, gender and tested by analysis of covariance (ANCOVA). In case of an allele dose effect, we performed a linear regression analysis to quantify association. Statistical significance was defined as a two-tailed $P < 0.05$.

RESULTS

The frequencies of the OATP1C1-intron3C>T, OATP1C1-Thr[143] and OATP1C1-3035T alleles were 46.1%, 3.2% and 42.9% respectively and similar to those found in public databases such as NCBI SNP database (http://www.ncbi.nlm.nih.gov) and the Hapmap Project (www.hapmap.org) (36) (Table 1). All polymorphisms were in Hardy-Weinberg equilibrium. No associations were found between the polymorphisms and serum thyroid parameters (data not shown).

OATP1C1 polymorphisms and scores on well-being questionnaires

Table 2 shows the scores on well-being questionnaires by genotype. OATP1C1-intron3C>T was significantly associated, in an allele dose-dependent manner, with 7 out of 8 fatigue-subscales in different questionnaires. Patients homozygous for this polymorphism were consistently least fatigued as indicated by their scores on all subscales of a questionnaire specifically designed to measure several aspects of fatigue (MFI-20), and on the subscales of other well-being questionnaires measuring fatigue (POMS fatigue) or the opposite (POMS vigor; Rand-36 vitality). Wild-type patients were most fatigued, and the fatigue-scores of patients heterozygous for the polymorphism were intermediate. In the same manner, this polymorphism was associated with the three subscales measuring symptoms of depression. Again, homozygous patients had the least complaints of depression indicated by lower scores, whereas wild-type patients had the highest scores on subscales of depression. The association was significant for one of the three subscales (SCL-90 depression), for the two other subscales (POMS depression and Rand mental health) there was a trend towards significance.

Like OATP1C1-intron3C>T, OATP1C1-C3035T was significantly associated with scores on all three questionnaire subscales measuring depression, again in an allele dose-dependent manner. Wild-type patients consistently had the worst depression scores (indicating more complaints of depression), whereas homozygous carriers were the least depressed. For instance, on the POMS depression subscale, wild-type patients had a mean score of 7.6 ± 1.0, compared to homozygous variant carriers who had an average score of 3.8 ± 1.3 (P=0.01).

Table 1: OATP1C1 genotype frequencies in a population of patients on T4 replacement therapy for primary autoimmune hypothyroidism

	Genotypes				
	wild-types	heterozygotes	homozygotes	allele frequency	P[a]
Intron3C>T	43	65	32	0.46	0.44
Pro143Thr	132	9	0	0.03	0.70
C3035T	46	69	26	0.43	0.99

[a] P for deviation from Hardy-Weinberg equilibrium

Table 2: Scores on well-being questionnaires by OATP1C1 genotype

		OATP1C1-intron3C>T				OATP1C1-Pro143Thr			OATP1C1-C3035T			
		wild-types	heterzygotes	homozygotes	P	wild-types	heterozygotes	P	wild-types	heterozygotes	homozygotes	P
	N	43	65	32		132	9		46	69	26	
POMS												
	Depression	6.8 ± 1.0	5.3 ± 0.8	4.1 ± 1.2	0.08	5.3 ± 0.6	7.3 ± 2.2	0.39	7.6 ± 1.0	4.6 ± 0.8	3.8 ± 1.3	0.01
	Fatigue	12.3 ± 1.0	10.1 ± 0.8	8.8 ± 1.2	0.02	10.1 ± 0.6	14.8 ± 2.2	0.05	11.2 ± 1.0	10.2 ± 0.8	9.6 ± 1.4	0.33
	Vigour	7.2 ± 0.6	9.1 ± 0.5	9.2 ± 0.7	0.02	8.7 ± 0.4	7.6 ± 1.4	0.43	8.1 ± 0.6	8.8 ± 0.5	8.8 ± 0.8	0.40
	Anger	7.1 ± 0.9	6.6 ± 0.7	6.5 ± 1.0	0.60	6.7 ± 0.5	6.8 ± 2.0	0.96	8.4 ± 0.8	6.2 ± 0.7	5.0 ± 1.2	0.01
	Tension	7.4 ± 0.9	6.0 ± 0.7	5.5 ± 1.0	0.16	6.3 ± 0.5	6.9 ± 1.9	0.74	7.6 ± 0.8	5.8 ± 0.7	5.4 ± 1.2	0.09
MFI												
	General fatigue	15.9 ± 0.6	15.5 ± 0.5	13.4 ± 0.7	0.02	15.0 ± 0.4	16.7 ± 1.4	0.25	15.2 ± 0.6	14.9 ± 0.5	15.3 ± 0.9	0.98
	Physical fatigue	14.0 ± 0.6	13.1 ± 0.5	11.9 ± 0.5	0.03	12.9 ± 0.4	14.9 ± 1.4	0.16	13.2 ± 0.6	12.8 ± 0.5	13.2 ± 0.8	0.96
	Reduced activity	12.7 ± 0.7	11.9 ± 0.6	10.0 ± 0.8	0.01	11.5 ± 0.4	13.8 ± 1.5	0.15	11.5 ± 0.7	11.5 ± 0.6	12.2 ± 0.9	0.58
	Reduced motivation	11.8 ± 0.7	11.3 ± 0.5	9.2 ± 0.8	0.01	10.9 ± 0.4	11.0 ± 1.5	0.94	10.9 ± 0.7	10.8 ± 0.5	11.3 ± 0.9	0.78
	Mental fatigue	13.1 ± 0.7	13.2 ± 0.6	11.2 ± 0.9	0.12	12.8 ± 0.4	11.8 ± 1.6	0.58	13.4 ± 0.7	12.5 ± 0.6	12.1 ± 1.0	0.27
RAND												
	Vitality	39.1 ± 3.1	42.3 ± 2.6	50.0 ± 3.6	0.03	44.1 ± 1.8	30.7 ± 6.9	0.06	41.5 ± 3.1	43.9 ± 2.5	44.6 ± 4.3	0.52
	Mental health	61.4 ± 2.9	65.0 ± 2.4	69.0 ± 3.4	0.09	70.0 ± 1.7	64.5 ± 6.5	0.95	61.4 ± 2.8	64.8 ± 2.3	71.8 ± 3.9	0.04
SLC-90												
	Depression	31.7 ± 1.7	29.4 ± 1.4	26.2 ± 2.0	0.04	29.1 ± 1.0	32.2 ± 3.8	0.44	32.2 ± 1.7	28.3 ± 1.4	26.8 ± 2.3	0.04
	Agoraphobia	8.7 ± 0.6	9.4 ± 0.5	8.6 ± 0.7	0.95	8.9 ± 0.4	9.3 ± 1.3	0.79	9.0 ± 0.6	9.2 ± 0.5	8.2 ± 0.8	0.52
	Anxiety	16.5 ± 1.0	16.0 ± 0.8	14.8 ± 1.2	0.26	15.9 ± 0.6	15.1 ± 2.2	0.72	16.9 ± 0.9	16.0 ± 0.8	13.5 ± 1.3	0.05
	Somatic complaints	24.9 ± 1.2	24.2 ± 1.0	23.1 ± 1.4	0.37	24.1 ± 0.7	24.4 ± 2.7	0.92	25.0 ± 1.2	24.4 ± 1.0	21.9 ± 1.6	0.17
	Insufficient functioning	20.2 ± 1.1	20.0 ± 0.9	17.1 ± 1.2	0.07	19.5 ± 0.6	18.1 ± 2.3	0.58	20.2 ± 1.0	19.2 ± 0.8	18.3 ± 1.4	0.26
	Paranoid ideation	30.3 ± 1.7	28.1 ± 1.4	26.8 ± 2.0	0.16	28.5 ± 1.0	28.3 ± 3.7	0.96	31.0 ± 1.6	28.1 ± 1.3	25.2 ± 2.2	0.04
	Hostility	8.7 ± 0.5	9.0 ± 0.4	8.3 ± 0.6	0.72	8.7 ± 0.3	8.6 ± 1.1	0.92	9.5 ± 0.5	8.4 ± 0.4	8.1 ± 0.7	0.07
	Sleeping	7.5 ± 0.5	7.3 ± 0.4	6.3 ± 0.6	0.18	7.1 ± 0.3	7.3 ± 1.2	0.89	7.2 ± 0.5	7.3 ± 0.4	6.4 ± 0.7	0.46
	Total score	162.0 ± 7.4	156.4 ± 6.1	144.1 ± 8.6	0.13	155.1 ± 4.3	156.1 ± 16.4	0.95	165.5 ± 7.1	153.7 ± 5.8	140.2 ± 9.9	0.04

Higher scores indicate more complaints, except for the POMS vigour and both Rand-36 subscales where lower scores indicate more complaints. Scales in italic are scales related to depression. Scales, which are underlined, are scales measuring fatigue.

Table 3: Scores on neurocognitive tests by OATP1C1 genotype

		OATP1C1-intron3C>T			
		wild-types	heterzygotes	homozygotes	P
	N	43	65	32	
Digit Symbol					
	Raw score (sec)	74.0 ± 2.8	71.7 ± 2.3	72.9 ± 3.2	0.75
MCT (paper & pencil version)					
	1 letter (sec)	27.6 ± 1.0	25.3 ± 0.8	26.2 ± 1.1	0.28
	2 letters (sec)	37.1 ± 1.4	36.6 ± 1.2	36.2 ± 1.6	0.65
	3 letters (sec)	45.8 ± 1.8	44.2 ± 1.5	45.8 ± 2.1	0.94
	4 letters (sec)	58.5 ± 2.4	55.2 ± 2.0	58.8 ± 2.8	0.95
MCT (computer version)					
	3 letters (sec)	621.7 ± 14.1	628.7 ± 11.8	638.0 ± 16.6	0.45
	4 letters (sec)	616.8 ± 12.1	628.6 ± 10.1	622.0 ± 14.2	0.72
	5 letters (sec)	678.9 ± 11.9	681.4 ± 10.0	677.6 ± 14.0	0.96
	Intercept (sec)	553.3 ± 21.1	567.4 ± 17.7	586.4 ± 24.8	0.31
	Slope	28.7 ± 5.0	26.3 ± 4.2	19.8 ± 5.9	0.26
Pasat					
	Total score (No)	223.6 ± 7.8	223.5 ± 6.5	212.3 ± 9.2	0.38
Digit Symbol					
	Pairs	12.4 ± 0.7	11.5 ± 0.5	11.0 ± 0.8	0.17
	Free reproduction	7.6 ± 0.2	7.3 ± 0.2	7.1 ± 0.2	0.11
Digit Span					
	Forward recall	8.6 ± 0.3	8.6 ± 0.2	8.2 ± 0.3	0.36
	Backward recall	5.9 ± 0.3	5.9 ± 0.3	5.4 ± 0.4	0.35
CVLT					
	Immediate recall (No)	51.8 ± 1.5	51.4 ± 1.2	50.1 ± 1.7	0.46
	Delayed recall (No)	12.2 ± 0.4	12.5 ± 0.4	11.4 ± 0.5	0.37
	Recognition (No)	1.1 ± 0.2	0.9 ± 0.2	1.5 ± 0.3	0.28
Rivermead (stories)					
	Immediate recall (No)	16.5 ± 1.0	16.9 ± 0.8	16.8 ± 1.1	0.80
	Delayed recall (No)	13.5 ± 1.0	14.1 ± 0.8	13.8 ± 1.1	0.79
	Proportion recalled (%)	78.3 ± 2.6	81.9 ± 2.1	81.0 ± 3.0	0.45

Table 4: Preference for LT4/LT3 therapy over LT4 treatment alone by OATP1C1 genotype

	OATP1C1-intron 3C>T		
Preference for LT4/LT3 over LT4	wild-types	heterozygotes	homozygotes
No (number of subjects and %)	10 (45.5)	25 (56.8)	13 (52.0)
Yes (number of subjects and %)	12 (54.5)	19 (43.2)	12 (48.0)
			0.68[a]

[a] P-value for χ^2 –test.

OATP1C1-Pro143Thr			OATP1C1-C3035T			
wild-types	heterozygotes	P	wild-types	heterozygotes	homozygotes	
132	9		46	69	26	
72.7 ± 1.6	74.4 ± 6.1	0.79	71.7 ± 2.7	73.6 ± 2.2	72.6 ± 3.6	0.01
26.1 ± 0.6	26.8 ± 2.2	0.77	27.4 ± 1.0	25.9 ± 0.8	24.8 ± 1.3	0.09
36.5 ± 0.8	38.7 ± 3.1	0.48	38.4 ± 1.3	36.1 ± 1.1	34.7 ± 1.8	0.09
44.9 ± 1.1	46.1 ± 4.0	0.79	47.8 ± 1.8	44.0 ± 1.4	42.9 ± 2.4	0.07
57.0 ± 1.4	57.3 ± 5.3	0.95	59.6 ± 2.3	55.7 ± 1.9	55.9 ± 3.2	0.27
628.1 ± 8.1	630.8 ± 30.7	0.93	635.0 ± 13.9	625.5 ± 11.3	623.7 ± 18.4	0.58
622.5 ± 7.0	635.8 ± 26.3	0.63	626.8 ± 11.9	618.5 ± 9.7	629.8 ± 15.8	0.99
680.1 ± 6.8	672.6 ± 25.9	0.78	682.1 ± 11.7	678.8 ± 9.6	677.6 ± 15.5	0.80
565.6 ± 12.2	583.7 ± 46.1	0.71	577.6 ± 20.9	561.2 ± 17.0	562.7 ± 27.6	0.61
26.0 ± 2.9	20.8 ± 10.9	0.65	23.5 ± 4.9	26.6 ± 4.0	27.0 ± 6.5	0.63
220.9 ± 4.5	226.6 ± 17.9	0.76	215.0 ± 7.6	224.6 ± 6.3	223.2 ± 10.3	0.43
11.7 ± 0.4	11.9 ± 1.5	0.88	11.4 ± 0.6	11.8 ± 0.5	12.1 ± 0.9	0.55
7.4 ± 0.1	7.3 ± 0.4	0.79	7.3 ± 0.2	7.4 ± 0.2	7.4 ± 0.3	0.87
8.5 ± 0.2	9.3 ± 0.7	0.27	8.3 ± 0.3	8.6 ± 0.2	8.7 ± 0.4	0.41
5.8 ± 0.2	6.3 ± 0.7	0.44	5.7 ± 0.3	6.1 ± 0.3	5.3 ± 0.4	0.60
50.9 ± 0.8	55.4 ± 3.2	0.18	49.8 ± 1.4	51.6 ± 1.2	52.4 ± 1.9	0.25
12.0 ± 0.3	13.6 ± 1.0	0.12	11.7 ± 0.4	12.2 ± 0.4	12.8 ± 0.6	0.11
1.1 ± 0.1	0.8 ± 0.5	0.57	1.2 ± 0.2	1.2 ± 0.2	0.8 ± 0.3	0.38
16.9 ± 0.5	14.1 ± 2.1	0.20	17.1 ± 0.9	16.6 ± 0.8	15.9 ± 1.2	0.48
13.9 ± 0.5	11.7 ± 2.1	0.30	14.1 ± 0.9	13.6 ± 0.8	13.8 ± 1.2	0.77
80.5 ± 1.5	81.3 ± 5.6	0.89	79.6 ± 2.5	80.1 ± 2.0	83.6 ± 3.4	0.39

OATP1C1-Pro143Thr			OATP1C1-C3035T		
wild-types	heterozygotes		wild-types	heterozygotes	homozygotes	
45 (51.7)	4 (80.0)		16 (55.2)	25 (53.2)	8 (50.0)	
42 (48.3)	1 (20.0)		13 (44.8)	22 (46.8)	8 (50.0)	
		0.22[a]				0.95[a]

Similar associations were found on the Rand mental health and the SLC90 depression subscales.

Concerning OATP1C1-Pro143Thr, carriers of the Thr^{143} allele (n= 9) had slightly worse scores on all but one of the 8 fatigue subscales, however the associations failed to reach significance.

OATP1C1 polymorphisms and scores on neurocognitive functioning tests

With regard to neurocognitive function, no significant associations were found between any of the polymorphisms and scores on neurocognitive functioning tests for cognitive speed, attention and memory (Table 3).

OATP1C1 polymorphisms and preference for LT4/LT3 therapy over T4 treatment alone

Data for T4/T3 preference were available for 140 out of 141 participants. After baseline measurements, 92 patients received combined LT4/LT3 therapy in a ratio of 5:1 or 10:1 for 15 weeks. The proportion of patients indicating at endpoint that they preferred study medication (i.e. combined LT4/LT3 treatment) over LT4 monotherapy prior to study entry was not significantly associated with the different genotypes (Table 4).

DISCUSSION

In this study of 141 patients with adequately treated hypothyroidism, we analyzed whether genetic variation in the brain-specific thyroid hormone transporter OATP1C1 is a determinant of well-being and neurocognitive functioning. Secondly, we investigated if OATP1C1 genotypes are associated with preference for combined LT4/LT3 therapy.

Two polymorphisms, spanning two large haploblocks in the OATP1C1 gene, were associated with symptoms of fatigue and depression in an allele dose-dependent manner. OATP1C1-intron3C>T was significantly associated with fatigue. Wild-type patients consistently displayed the highest levels of symptoms, and homozygous variant carriers the lowest levels. The same allele dose-dependent association was observed with the subscales measuring depression. In addition, OATP1C1-C3035T was associated with depression. Again, wild-type patients had the highest levels of symptoms whereas homozygous variant carriers reported the least depression.

The extent of the effect of OATP1C1 genotypes on measurements of well-being is best illustrated with an example. The mean SCL-90 depression subscale scores in our study population were 29.3. In the general population scores in this range (25-35) are labeled as 'high' meaning that these scores are between the 80^{th} and 95^{th} percentile (3). Regarding OATP1C1-C3035T, wild-type patients had a mean score of 32.2 on the SLC-90 depression subscale, whereas homozygote carriers of the 3035T-allele had an average score of 26.8 (Table 2). Whether one

should consider this to be a clinically relevant effect is open to debate. Single polymorphisms generally have a subtle effect, while clinically significant effects usually result from the addition of cumulative effects of many genes each contributing only slightly to the variance. Thus, genetic variation in OATP1C1 is one of many determinants of well-being. The associations between polymorphisms in OATP1C1 and symptoms like depression strengthen the notion that OATP1C1 is important for thyroid hormone transport across the blood-brain barrier.

One may hypothesize that, if the delivery of T4 across the blood-brain barrier by OATP1C1 is decreased due to polymorphisms in OATP1C1, these patients would benefit from combined therapy with both LT4 and LT3 through adding a direct source of T3. Nonetheless, in this study we found no association between OATP1C1 genotypes and a preference for combined treatment. However, no data are available about the extent to which exogenous T3 is effectively transported into different brain regions in man, while only sparse data are present from animal experimental studies (37).

No associations were found between OATP1C1 genotypes and scores on neurocognitive functioning tests. This is in concordance with the fact that, as we have shown in an earlier publication, the SCL-90 total score, regarded as the main summarizing measure of well-being in this study, was only weakly correlated with some, but not all of the neurocognitive test results (3). In fact, the determinants of decreased levels of well-being on the one hand, and impaired neurocognitive functioning on the other, may well be of a different origin.

This is the first study to find associations between polymorphisms in the brain-specific thyroid hormone transporter OATP1C1 and scores on well-being questionnaires in adequately treated hypothyroid patients. However, limitations of our study have to be mentioned. For instance, the four questionnaires chosen to investigate different aspects of well-being all had several subscales, 21 subscales in total. Due to multiple comparisons this increases the possibility of type I errors, warranting cautious interpretation of the results. However, the consistency and specificity of the associations with fatigue and depression are highly supportive of the validity of these findings. For example, 8 out of the 21 subscales address symptoms of fatigue and on 7 of these 8 subscales a significant dose-allele relation was found for OATP1C1-intron3C>T (Table 2). Similarly, a significant and allele-dose dependent association between OATP1C1-C3035T and symptoms of depression was found for all three subscales addressing these symptoms (Table 2).

In summary, in a population of patients with adequately treated hypothyroidism OATP1C1 polymorphisms are associated with fatigue and depression, but they do not explain differences in neurocognitive functioning or preference for LT4/LT3 combination therapy. Further studies are needed to confirm our findings and to elucidate the determinants of neurocognitive impairments and a preference for combined LT4/LT3 treatment. In addition, demonstration of a functional effect of these polymorphisms on the OATP1C1 protein is necessary.

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Chapter 5

Thyroid hormone transport by OATP1A2 and OATP1B3: functional comparison with other human OATP1 family members

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Submitted

ABSTRACT

As a prerequisite for intracellular processes like deiodination, sulfoconjugation or binding to the thyroid hormone receptor, thyroid hormone is actively transported across the cell membrane by thyroid hormone transporters, such as members of the organic anion transporting polypeptide family (OATPs). We have previously demonstrated that OATP1B1 strongly induces uptake of iodothyronine sulfates T4 sulfate (T4S), T3 sulfate (T3S) and reverse T3 sulfate (rT3S), but has no T4 and T3 and only little rT3 transport activity. OATP1C1 transports T4 and rT3, and to some extent T4S. In this study, we examined the specificity of iodothyronine transport by the two remaining human members of the OATP1 subfamily, OATP1A2 and OATP1B3, by analysis of iodothyronine uptake and metabolism.

Functional expression studies in *Xenopus laevis* oocytes and COS1 cells indicate that OATP1A2 significantly induced uptake of T4, T3, rT3, T4S, T3S and rT3S. In addition, metabolism of these substrates by co-transfected deiodinases was greatly augmented in the presence of OATP1A2. OATP1B3, however, predominantly transports rT3 and the sulfated iodothyronines. Metabolism of these substrates by type 1 deiodinase (D1) was markedly increased when cells were co-transfected with OATP1B3.

In conclusion, these four human members of the OATP1 subfamily exhibit overlapping substrate specificities. OATP1A2 is unique in transporting all (sulfated) iodothyronines, whereas OATP1C1 has much narrower substrate specificity. OATP1B1 and OATP1B3, both expressed exclusively in the liver, preferentially transport rT3 and iodothyronine sulfates, probably facilitating their degradation by liver D1.

INTRODUCTION

As a prerequisite for intracellular processes like deiodination, sulfoconjugation or binding to its nuclear receptor (1-3), thyroid hormone is actively transported across the cell membrane by thyroid hormone transporters. Several proteins can facilitate this transport, such as members of the organic anion transporting polypeptide family (OATPs) (4), amino acid transporters (5) or monocarboxylate transporters (6).

OATPs are a large family of multi-specific sodium-independent transport proteins that are expressed in many tissues (Fig. 1A). Among the many substrates transported by OATPs are thyroid hormones (7-11). All four human members of the OATP1 subfamily, OATP1A2, OATP1B1 and OATP1B3 and OATP1C1, are located in a gene cluster on chromosome 12p12,

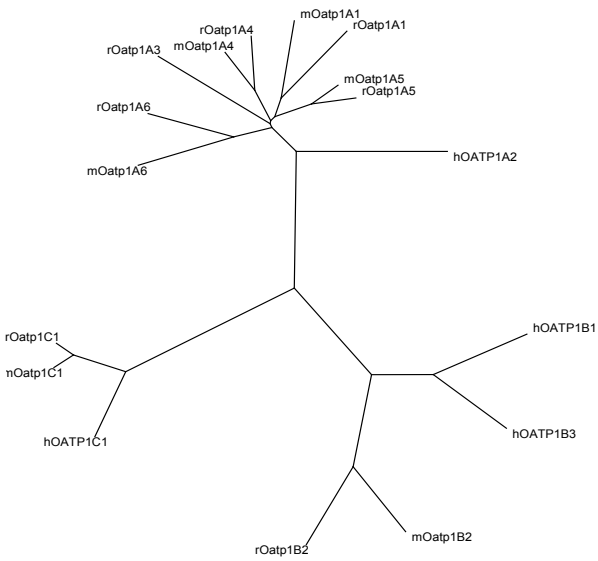

Figure 1A: Phylogenetic tree of human and rodent (rat and mouse) members of the OATP1 subfamily. Multiple sequence aligment of amino acid sequences and phylogenetic tree construction were carried out using Clustal W (http://www.ebi.ac.uk/clustalw). The phylogenetic tree was made by Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). This figure is based on Genebank accession numbers NM013797 (mOATP1A1), NM017111 (rOATP1A1), NM134431 (hOATP1A2), NM030837 (rOATP1A3), NM030687 (mOATP1A4), NM131906 (rOATP1A4), NM130861 (mOATP1A5), NM030838 (rOATP1A5), NM023718 (mOATP1A6), NM130736 (rOATP1A6), NM006446 (hOATP1B1), NM020495 (mOATP1B2), NM031650 (rOATP1B2), NM019844 (hOATP1B3), NM017435 (hOATP1C1), NM021471 (mOATP1C1), NM053441 (rOATP1C1).

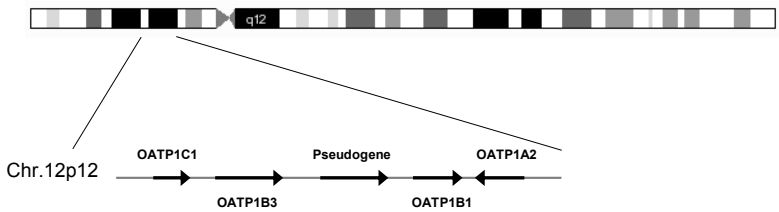

Figure 1B: Overview of the genomic structure of the human OATP1 cluster on chromosome 12p12.

together with a pseudogene (Fig. 1B). These OATPs have a similar genomic organization, i.e. in all four transporter genes the open reading frame is composed of 14 exons. In addition, they share on average almost 50% amino acid identity and are considered to be 12 transmembrane domain glycoproteins (4, 11).

Recently, we have shown that OATP1B1 strongly induces uptake of iodothyronine sulfates T4 sulfate (T4S), T3 sulfate (T3S) and reverse T3 sulfate (rT3S), but has only little T4, T3 and rT3 transport activity (12). A different member of the OATP1 subfamily, OATP1C1, shows high substrate specificity as it only transports T4 and rT3, and to some extent also T4S (11, 13).

In this study, we examined the specificity of iodothyronine transport by OATP1A2 and OATP1B3 and analyzed whether these transporters are rate-limiting in subsequent iodothyronine metabolism in cells co-transfected with different deiodinases. In addition, these transporter proteins were functionally compared with OATP1B1 and OATP1C1, the other two human members of the OATP1 subfamily.

MATERIALS AND METHODS

Materials

FuGENE6 transfection reagent was obtained from Roche Diagnostics (Indianapolis, USA). [^{125}I]NaI, [^{125}I]T4 and [^{125}I]T3 were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). [^{125}I]rT3 was obtained from Perkin Elmer (Boston, MA). Unlabeled T4, T3 and rT3 were bought from Henning GmbH (Berlin, Germany). [^{125}I]T4S, [^{125}I]T3S and [^{125}I]rT3S were synthesized as described previously (14).

Plasmids

The pSPORT1-OATP1A2 plasmid was kindly provided by Prof. Dr. Peter J. Meier (Institute of Clinical Pharmacology and Toxicology, University Hospital Zürich, Switzerland). OATP1A2

was subcloned into pSG5 (Stratagene, La Jolla, USA) using *Not*I and *Acc65*I restriction sites. Prof. Dr. Dietrich Keppler (German Cancer Research Center, Heidelberg, Germany) provided us with the pcDNA3.1-Hygro-OATP1B3 plasmid. OATP1B3 was subcloned into pSG5 via a *BstX*1 restriction site. pSG5-μ-crystallin (CRYM), pcDNA3-ratD1 (rD1) and pCIneo-D3 plasmids were constructed as previously described (15).

For the experiments with *X. laevis* oocytes, the pSPORT1-OATP1A2 plasmid was linearized with the restriction enzyme *Xba*I to create 5'-protroding ends, and transcribed using the Ampliscribe High Yield T7 RNA transcription kit (Epicentre Technologies, Madison, WI). The cRNA was capped with the m7G (5')ppp(5')G cap analog (Epicentre) and stored in RNAase free water at -80 C.

COS1 cell culture

COS1 cells were cultured in 6 or 24-well dishes (Corning, Schiphol, The Netherlands) with DMEM/F12 medium (Invitrogen), containing 9% heat-inactivated fetal bovine serum (Invitrogen) and 100 nM sodium selenite (Sigma).

Iodothyronine (sulfate) transport and metabolism in COS1 cells

Uptake studies: COS1 cells were cultured in 6-well culture dishes, and transfected in duplicate with 500 ng pSG5 plasmid without or with OATP1A2 or OATP1B3 using FuGENE6 according to the manufacturer's guidelines. Cells were co-transfected with 500 ng pSG5-CRYM, coding for a high-affinity cytosolic thyroid hormone-binding protein that prevents efflux of internalized iodothyronines (16). This protein binds not only T4 and T3, but also T4S and T3S (personal communication W.E. Visser). The affnity of CRYM for rT3 and rT3S is much lower compared to the other iodothyronines (12, 13). After 48 h, cells were washed with incubation medium (Dulbecco's PBS, 0.1% BSA and 0.1% glucose), and incubated for 5, 10 or 30 min at 37 C with 1 nM ($2x10^5$ cpm) ^{125}I-labeled T4, T3, rT3, T4S, T3S or rT3S in 1.5 ml incubation medium. After incubation, cells were harvested and analyzed as described previously (15).

Metabolism studies: COS1 cells were cultured in 24-well culture dishes and transfected with 100 ng pCIneo-D3 or pcDNA3-rD1 and 100 ng pSG5-OATP1A2 or pSG5-OATP1B3. 48 h after transfection, cells were incubated for 4 or 24 h at 37 C with 1 nM ($1x10^6$ cpm) ^{125}I-labeled T4, T3, rT3, T4S, T3S or rT3S in 0.5 ml incubation medium. After incubation, medium was harvested and analyzed by HPLC as described previously (15).

Iodothyronine transport in X. laevis oocytes

Oocytes were prepared as described previously (17). After isolation, oocytes were sorted on morphological criteria and defolliculated manually. Healthy-looking stage V–VI oocytes were kept at 18 C in modified Barth's solution containing 20 IU/ml penicillin and 20 μg/ml streptomycin. The next day, oocytes were injected with 2.3 ng OATP1A2 cRNA in 23 nl water

using the Nanoject system (Drummond Scientific, Broomall, PA). Uninjected oocytes were used as controls, as similar results were obtained using water-injected oocytes. Injected and uninjected oocytes were kept for 3-4 d at 18 C in modified Barth's solution.

Uptake assays were performed as reported previously (17). Groups of 10 oocytes were incubated for 1 h at 25 C with 100 nM [^{125}I]T4, [^{125}I]T3 or [^{125}I]rT3 in 0.1 ml incubation medium [100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM Tris, pH 7.5]. After incubation, oocytes were washed 4 times with 2.5 ml ice-cold incubation medium containing 0.1% BSA. Oocytes were transferred to new tubes and counted individually.

RESULTS

Iodothyronine (sulfate) transport by OATP1A2 and OATP1B3 in transfected COS1 cells

OATP1A2 significantly induced uptake of all iodothyronines tested in this study (Fig. 2). After 30 min incubation, T4, T3 and rT3 uptake were almost 2-fold higher in OATP1A2 transfected cells than in mock transfected cells. In addition, OATP1A2 clearly induced iodothyronine sulfate uptake: in OATP1A2 transfected cells the uptake of T4S and T3S was almost 10 times higher than in cells transfected with empty vector. This also holds true for rT3S, with a 4-fold higher induction in uptake by OATP1A2 compared with empty vector.

In contrast to OATP1A2, OATP1B3 did not or only poorly enhance T4 and T3 uptake. However, transport of rT3 and the iodothyronine sulfates T4S, T3S and rT3S was clearly induced by OATP1B3.

Iodothyronine (sulfate) metabolism by OATP1A2 and OATP1B3 in transfected COS1 cells

Cells were transfected with D3 for the metabolism experiments with T4 and T3, since T4 and T3 are good substrates for D3 (3). For the metabolism assays with rT3, we co-transfected OATP1A2 or OATP1B3 with D1 as rT3 is the preferred substrate of this deiodinase. This also holds for iodothyronine sulfates which are are only deiodinated by D1.

Incubation of cells transfected with D1 or D3 alone already led to some conversion of T4, T3 and rT3 (Fig. 3). However, this was markedly and significantly increased, when cells were co-transfected with OATP1A2 (Fig. 3). In a similar manner, we found that metabolism of T4S, T3S and rT3S by D1 was clearly augmented by co-transfection of OATP1A2.

Co-transfection of cells with OATP1B3 increased metabolism of T4, but not of T3, by D3. In addition, metabolism of rT3 and iodothyronine sulfates by D1 was significantly increased when OATP1B3 was co-transfected (Fig. 3). About 40% of T4S, 10% of T3S and 40% of rT3S was deiodinated by D1 when OATP1B3 was co-transfected, whereas no iodide was produced in cells transfected with D1 alone (Fig. 3).

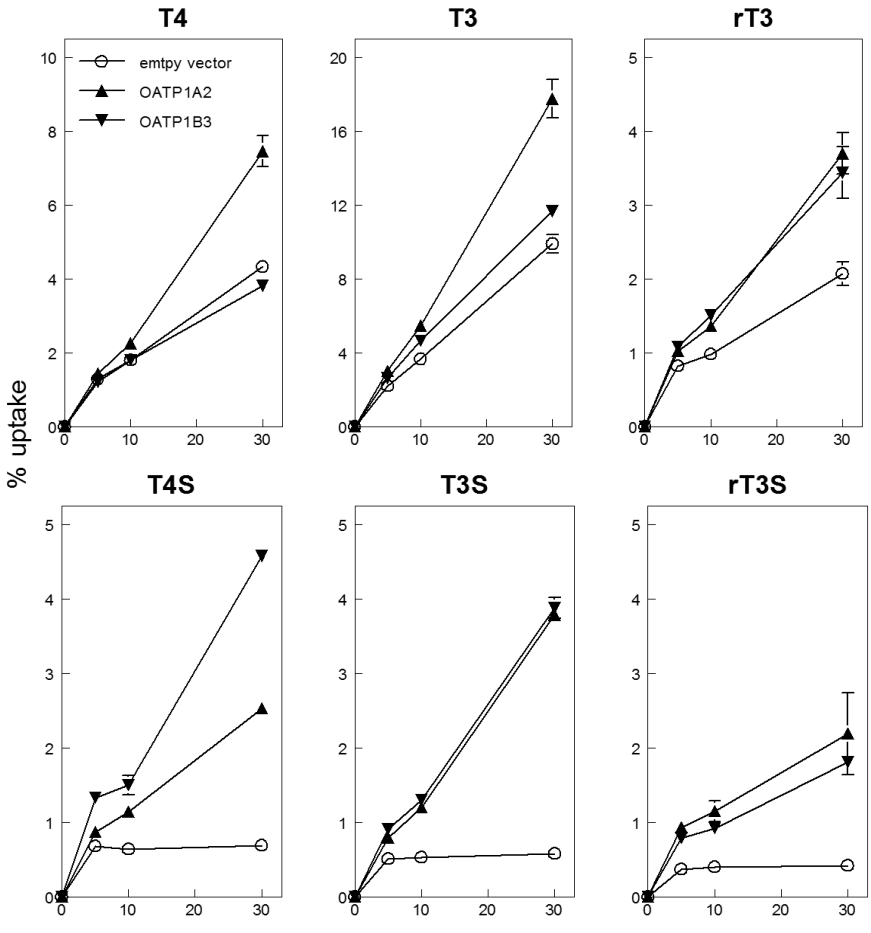

Figure 2; [^{125}I]T4, [^{125}I]T3, [^{125}I]rT3, [^{125}I]T4S, [^{125}I]T3S and [^{125}I]rT3S uptake by COS1 cells transfected with empty vector, OATP1A2 or OATP1B3. Cells were co-transfected with CRYM, an intracellular thyroid hormone-binding protein. Cells were incubated for 5, 10 or 30 min at 37 C with 1 nM ($2x10^5$ cpm) ^{125}I-labeled T4, T3, rT3, T4S, T3S or rT3S. Data are expressed as percentage uptake of added radioactivity. Data are presented as the means ± SEM of three experiments.

Iodothyronine transport by OATP1A2 in X. laevis oocytes

Analogous to the results of the experiments in transiently transfected COS1 cells, oocytes expressing OATP1A2 clearly showed induced uptake of all iodothyronines tested (Fig. 4). After 1 h incubation, T4, T3 and rT3 uptake was 1.5 to 1.9-fold increased in OATP1A2 expressing oocytes compared with uninjected oocytes.

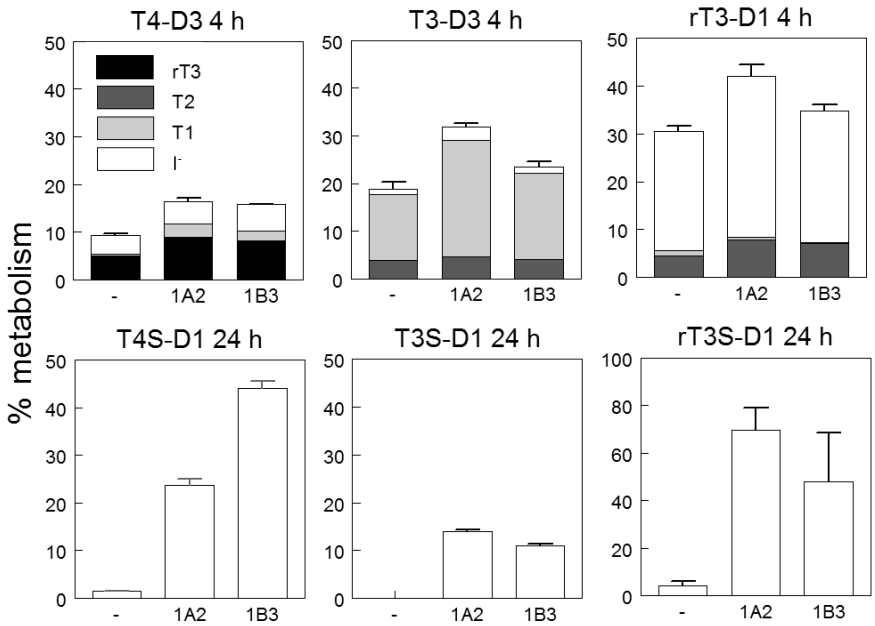

Figure 3: [^{125}I]T4, [^{125}I]T3, [^{125}I]rT3, [^{125}I]T4S, [^{125}I]T3S and [^{125}I]rT3S metabolism by COS1 cells transfected with either D1 or D3 alone or D1 or D3 together with OATP1A2 or OATP1B3. Cells were incubated for 4 h at 37 C with 1 nM (1x10^6 cpm) ^{125}I-labeled T4, T3 or rT3. Cells were incubated for 24 h at 37 C with 1 nM (1x10^6 cpm) ^{125}I-labeled T4S, T3S or rT3S. Metabolism is shown as percentage of metabolites in the medium after incubation for the indicated time period. Data are presented as the means ± SEM of at least three experiments. Data for [^{125}I]rT3S metabolism are the means ± SEM of two experiments.

Functional comparison of OATP1A2, OATP1B1, OATP1B3 and OATP1C1

Figure 5 gives an overview of iodothyronine uptake by the four human OATP1 subfamily members. For all experiments, cells were co-transfected with CRYM. CRYM binds T4, T3, T4S and T3S very well, but it has a low affinity for rT3 and rT3S. Therefore, rT3 and rT3S are not included in Figure 5.

It becomes apparent that OATP1A2 facilitates uptake of all (sulfated) iodothyronines, whereas OATP1C1 only transports T4 and T4S in a significant manner. OATP1B1 and OATP1B3 show similar preference for sulfated versus non-sulfated iodothyronines as ligands.

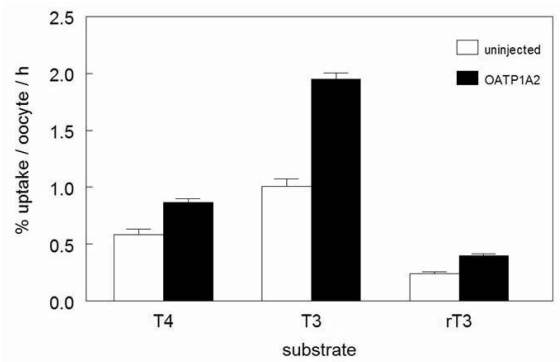

Figure 4: [^{125}I]T4, [^{125}I]T3 and [^{125}I]rT3 uptake by *X. laevis* oocytes uninjected or injected with OATP1A2. Oocytes were incubated for 1 h at 25 C with 100 nM (4x10^5 cpm) ^{125}I-labeled T4, T3 or rT3. Data are expressed as percentage uptake per oocyte of added radioactivity. Data are presented as the means ± SEM of 9-10 oocytes.

DISCUSSION

Our study indicates that all four human members of the OATP1 subfamily can transport iodothyronines. OATP1A2 is unique in transporting all sulfated and nonsulfated iodothyronines, whereas OATP1C1 has much narrower substrate specificity. OATP1B1 and OATP1B3 preferentially transport rT3 and iodothyronine sulfates.

Until now, several transporter families have been identified, among which the OATP family. OATPs mediate sodium-independent transport of a variety of organic compounds, such as bile acids, steroid hormones and numerous drugs (4). Different OATP family members have been extensively studied, as they take up endo- and xenobiotics from the blood into the liver, where they are transformed into water-soluble products that can be excreted into the bile (18). For instance, polymorphisms leading to decreased function of these transporter proteins have been associated with increased systemic bioavailability of drugs (19, 20). In this study, we examined the specificity of iodothyronine transport by OATP1A2 and OATP1B3. In addition, we compared these transporters with OATP1B1 and OATP1C1, the other two human members of the OATP1 subfamily that transport iodothyronines.

Functional expression studies in transfected COS1 cells indicate that OATP1A2 significantly induced uptake of T4, T3, rT3, T4S, T3S and rT3S. In addition, metabolism of these substrates by co-transfected deiodinases was greatly augmented in the presence of OATP1A2. Similar results regarding uptake of T4, T3 and rT3 by OATP1A2 were obtained in injected *X. laevis* oocytes, even though there are numerous differences between these two transport assays. First of all, there are clear differences in transfection efficiency. Every tested oocyte is

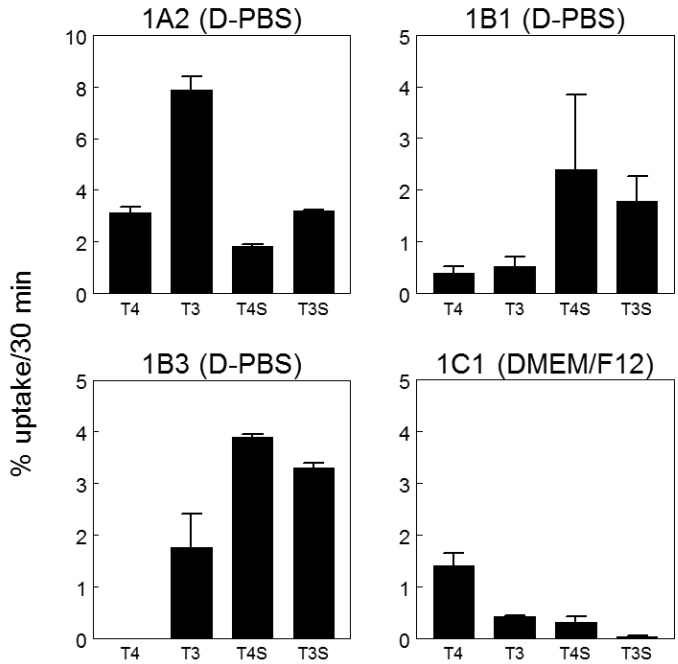

Figure 5: [^{125}I]T4, [^{125}I]T3, [^{125}I]T4S and [^{125}I]T3S uptake by COS1 cells transfected with one of the human OATP1 subfamily members. Cells were co-transfected with CRYM, an intracellular thyroid hormone-binding protein. Cells were incubated for 30 min at 37 C with 1 nM (2x10^5 cpm) ^{125}I-labeled T4, T3, T4S or T3S. All incubations were performed in Dulbecco's PBS with 0.1% BSA and 0.1% glucose, except for experiments with OATP1C1, which were performed in DMEM/F12 incubation medium supplemented with 0.1% BSA. Data were corrected for background uptake by subtraction of radioactivity taken up by cells transfected with empty vector instead of OATP. Data are presented as the means ± SEM of three experiments.

injected with cRNA coding for the protein of interest, whereas COS1 cells only show about 25% transfection efficiency. In addition, *X. laevis* oocytes have a higher intracellular thyroid hormone-binding capacity compared to COS1 cells. This difference was partially overcome by co-transfection with CRYM, a high-affinity cytosolic thyroid hormone-binding protein (16), which has recently been shown to greatly augment net T3 and T4 uptake by inhibition of T3 and T4 efflux (21). CRYM shows high affinity for T4 and T3, but also binds T4S and T3S. However, it has to be noted that the affnity of CRYM for rT3 and rT3S is much lower compared to the other iodothyronines (12). Finally, in the experiments performed with oocytes no albumin was present in the incubation medium and 100 nM of substrate was used. The incubation medium of COS1 cells was, however, supplemented with 0.1% BSA and a more physiological concentration of substrates, e.g. 1 nM, was used. Since similar results were obtained regarding

iodothyronine uptake by OATP1A2 in these different transport assays, it is clear that OATP1A2 indeed facilitates transport of thyroid hormones.

OATP1A2 is expressed in multiple tissues, among which liver, brain and kidney (8, 22). Based on its expression pattern, OATP1A2 could play a role in the delivery of thyroid hormone across the blood-brain barrier. Alternatively, it could also play a role in the removal of thyroid hormone from different tissues for degradation and eventual elimination via bile or urine.

OATP1B3 is exclusively expressed in liver (23). In transfected COS1 cells, OATP1B3 preferentially transports rT3 and the sulfated iodothyronines. In addition, metabolism of these substrates by D1 was markedly increased when cells were co-transfected with OATP1B3. It is thought that D1 in the liver under normal conditions rapidly degradates rT3 and sulfated iodothyronines, like T4S, T3S and rT3S, keeping the serum concentrations of these substrates low (1, 3, 24-27). It is, therefore, of interest that OATP1B3 only facilitates transport of these substrates, probably reflecting its role of transporter of endo- and xenobiotics that are metabolized in the liver and excreted in the bile. Moreover, OATP1B1, which has 80% amino acid sequence identity to OATP1B3 and is also exclusively expressed in the liver (4), showed identical substrate specificity (12).

OATP1C1, the fourth human member of the OATP1 subfamily, only transported T4 and rT3, and to some extent also T4S (11, 28). Based on the expression of OATP1C1 in capillaries in multiple brain regions, this protein is thought to play an important role in delivering serum thyroid hormone to the brain (11, 29). Additional support for a significant role for OATP1C1 in thyroid hormone transport in the brain comes from a study from Sugiyama and co-workers, in which they show that OATP1c1 is up-regulated in hypothyroid rats and down-regulated in hyperthyroid rats (29). Moreover, we have recently shown that polymorphisms in the OATP1C1 gene are associated with fatigue and depression in a cohort of hypothyroid patients (30).

In this study, we show that iodothyronine sulfates are novel substrates for OATP1A2 and OATP1B3. To more fully characterize their transport, Michaelis-Menten kinetics could be determined. However, this would not yield reliable Km values because of interfering factors. For instance, all experiments were performed in medium containing 0.1% BSA and all cells were co-transfected with CRYM, which have their own affinity for the iodothyronine sulfates. Therefore, we refrained form performing these experiments.

In conclusion, these four human members of the OATP1 subfamily exhibit overlapping substrate specificities. OATP1A2 transports all (sulfated) iodothyronines, whereas OATP1C1 has a much narrower substrate specificity. OATP1B1 and OATP1B3, both expressed exclusively in the liver, preferentially transport rT3 and iodothyronine sulfates, probably facilitating their degradation by liver D1.

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Chapter 6

The impact of a TSH receptor gene polymorphism on thyroid related phenotypes in a healthy Danish twin population

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ABSTRACT

Objective: The Asp727Glu polymorphism in the thyrotropin receptor (TSHR) gene is associated with serum TSH levels. However, the proportion of genetic variation accounted for by this polymorphism is unknown. In this study, we 1) examine the association of the Asp727Glu polymorphism with thyroid size, serum levels of TSH, thyroid hormones, and thyroid antibodies in 1241 healthy Danish twin individuals and 2) assess the contribution of the polymorphism to the trait variation and the genetic variance.

Measurements: The effect of the genotype on the traits (mean±SD) was established; associations between the TSHR-Asp727Glu polymorphism and measures of thyroid homeostasis were assessed and the effect of the polymorphism on the trait's phenotypic variability was quantified by incorporating the genotype information in structural equation modeling.

Results: The genotype distribution was Asp/Asp 84.9%; Asp/Glu 14.5% and Glu/Glu 0.6%. Carriers of the TSHR-Glu727 allele had lower TSH levels (non-carriers vs. carriers: 1.78±0.93 vs. 1.60±0.84mU/l, p=0.04). Regression analysis showed an association between the TSHR-Asp727Glu polymorphism and serum TSH (p=0.007). The polymorphism accounted for 0.91% of the total phenotypic variance in serum TSH levels. Including the genotype in quantitative genetic modeling improved model fit (p=0.001); however, the genetic influence on serum TSH not attributable to this specific genetic variant was only reduced from 68.2% to 67.8%. The polymorphism was not significantly associated with thyroid size, thyroid hormones or thyroid antibody levels.

Conclusion: The TSHR-727Glu allele was associated with decreasing TSH levels; however, the contribution to the genetic variance was very small. No association was found with other thyroid related measures.

INTRODUCTION

The circulating levels of serum thyroid stimulating hormone (TSH) and thyroid hormones represent biochemical phenotypes reflecting thyroid homeostasis. It is the combined effect of genetic and environmental factors that gives rise to these endophenotypes. Various groups (1-4) including our own (5-8) have previously established that the measures reflecting thyroid homeostasis have a substantial heritable component. However, the genetic variants underlying the genetic contribution to each of these traits are so far largely unknown. By including measured genotypes (for which an association has been established) into quantitative genetic models, it is possible to identify genetic variants that affect the variability as well as the hereditary component of a trait (9).

An obvious candidate gene involved in the control of the pituitary-thyroid axis is the TSH receptor (TSHR) gene, which is located on chromosome 14q31 (10). A variety of activating as well as inactivating mutations have been identified within the TSHR gene (11, 12). One of the best-studied polymorphisms within the TSHR gene is the Asp727Glu polymorphism. A C-G transition at position 2281 within codon 727 in this gene results in the aspartate to glutamate substitution within the cytoplasmic tail of the TSHR (13). Using a cohort of 156 healthy Dutch Caucasian blood donors, we recently found that the TSHR-Glu727 allele was associated with lower plasma TSH levels (10). However, initial positive findings in genetic association studies have proved to be difficult to confirm due to a large number of false positive findings, and cautious replication of promising findings are required (14-16).

Using the exact same polymorphism as in our initial studies, the current study was undertaken to evaluate the association of the Asp727Glu polymorphism in the TSHR gene and quantitative measures of thyroid homeostasis in a much larger and independent healthy Danish twin population. Moreover, by integrating measured environmental exposures such as iodine intake and cigarette smoking in the analyses, we examined whether the effect of the genotype on the phenotype was influenced by the presence of such environmental risk factors. Finally, using quantitative trait analysis we wanted to quantify the effect of this particular polymorphism on the genetic variation.

MATERIALS AND METHODS

Subjects

The present study is part of a nationwide project (GEMINAKAR) investigating the relative influence of genetic and environmental factors on a variety of different traits among Danish twins. Based on a questionnaire survey concerning physical health and health related behavior performed in 1994, a representative sample of complete twin pairs was recruited from the population-based Danish Twin Registry (17). A detailed description of the ascertainment

procedure can be found elsewhere (5, 8). Briefly, the twins included in the GEMINAKAR study were healthy as reported by themselves. With the exception of contraceptives, no twins were taking medicine known to affect the pituitary-thyroid axis or thyroid size. In order to get an equal distribution of twin pairs, sampling was stratified according to age, sex, and zygosity. The twins in a pair were examined on the same day. All twins with at least one partner living in the Western part of Denmark were examined in Odense, whereas all those where both partners were living in the Eastern part of Denmark were examined in Copenhagen. With the exception of 39 twin pairs, both twins in a pair lived in the same geographic region of Denmark. Blood and urine samples as well as DNA were collected between 8 and 9 a.m. after a twelve-hour fast. Thereafter, a clinical examination was performed and the twins filled in health related questionnaires including questions regarding thyroid disease, smoking habits and medicine intake.

In the GEMINAKAR study 1512 individuals (756 twin pairs) were examined. Blood samples for thyroid measurements were available from 1473 individuals. Serum TSH, serum free thyroxine (free T4), serum free triiodothyronine (free T3), serum thyroid peroxidase antibodies (TPOab) and thyroglobulin antibodies (Tgab) were measured. In all, DNA from 1079 individuals was genotyped. Due to problems with identification and genotyping, 17 and 18 individuals, respectively, were excluded leaving l044 individuals distributed in 289 monozygotic (MZ), 515 same sex dizygotic (DZ) and 240 opposite sex (OS) twin individuals. In 277 MZ twin pairs, in which only one of the twins was genotyped, we assumed identical genotypes. Thus, genotype information was available in 1321 individuals (566 MZ, 515 DZ, 240 OS) within 283 MZ, 269 DZ and 121 OS twin pairs. Twin pairs in which one or both twins had self-reported thyroid disease or overt biochemical thyroid disease were excluded (80 persons within 42 twin pairs), leaving 1241 (536 MZ, 477 DZ and 228 OS twin individuals) healthy, euthyroid individuals within 268 MZ, 248 DZ and 115 OS twin pairs. 593 complete twin pairs were without subclinical thyroid disease, and 531 twin pairs were thyroid antibody negative. Within the 1241 healthy twin individuals, 469 individuals had a thyroid ultrasound performed as previously described (5). Spot urine samples were available from 678 individuals.

Written informed consent was obtained from all participants and the study was approved by all regional Danish Scientific-Ethical Committees (case file 97/25 PMC).

Assays

Serum TSH was measured using a time-resolved fluoroimmunometric assay (AutoDELFIA hTSH Ultra Kit, Perkin Elmer/Wallac, Turku, Finland). Reference range is 0.30-4.00 mU/l. Serum free T4 and serum free T3 were determined using the AutoDELFIA FT4 and FT3 (Perkin Elmer/ Wallac, Turku, Finland), respectively. For free T4 the reference range is 9.9-17.7 pmol/l, and for free T3 it is 4.3-7.4 pmol/l. TPOab and Tgab were measured by solid phases, two step, time-resolved fluoroimmunoassays (AutoDELFIA TPOab kit and hTgab kit, respectively, Perkin Elmer/ Wallac, Turku; Finland). Values above 60 kIU/l were regarded as positive for TPOab as

well as for Tgab. Twin pairs were analyzed within the same run. All the serum samples were analyzed at the same laboratory in Odense. Zygosity was confirmed by analysis of nine highly polymorphic restriction fragment length polymorphisms and microsatellite markers widely scattered through the genome with an Applied Biosystems AmpFISTR Profiles Plus kit (The Perkin-Elmer corporation) (18).

The iodine concentration in the spot urine samples was analyzed by the cerium/arsenic method after digestion by alkaline ashing as described elsewhere (19). Individuals examined in Copenhagen (and therefore living in the Eastern part of Denmark) had a significantly higher iodine excretion than the twins examined and living in the Western part of Denmark (median 93.5 µg/l versus 71.0 µg/l). These results are in accordance with previous Danish epidemiological studies (19). According to WHO criteria (20), the Western part of Denmark can be classified as moderately iodine-deficient, and the Eastern part as mildly iodine deficient. Therefore, examination place was regarded as a marker for iodine intake.

Genotyping

Participants were genotyped for the TSHR-Asp727Glu polymorphism (rs1991517). Genomic DNA was isolated form peripheral leucocytes using standard procedures. Genotypes were determined in 10 ng genomic DNA using the Taqman allelic discrimination assay (Applied Biossytems, Nieuwerkerk a/d Ijssel, The Netherlands). Primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems. Reactions were performed on the Taqman 7900HT in 384-wells format in 2 µl reaction volume.

Statistical methods

Association between genotype and phenotype

These statistical analyses were carried out using STATA statistical software (21).

The distribution of thyroid size and serum TSH, TPOab, and Tgab levels was skewed and therefore transformed by the natural logarithm. Initially, the TSHR-Asp727Glu polymorphism was defined as a 3-level variable representing the 3 possible genotypes (homozygous non-carrier, heterozygous, and homozygous variant). However, only few individuals were homozygous for the TSHR-Glu727 allele, and they were therefore combined with the heterozygous group in the analyses assuming a dominant model - i.e. non-carriers of the TSHR-Glu727 allele were compared with carriers. The effect of the genotype on the trait means was established. Results are presented as means ± SD. A modified Wilcoxon test was used testing the differences between the groups (22). Moreover, associations between the Asp727Glu polymorphism in the TSHR gene and measures of thyroid homeostasis in the whole population were assessed using regression analysis. The statistical inference measures (i.e. p-values and CI) were computed using a technique (the cluster option in STATA), which takes the dependency of twin data into account. The genotype information was incorporated

in the regression analyses by coding the non-carriers as 0 and carriers as 1. In these analyses, adjustment for age and gender was performed.

To test whether the genotype-phenotype relationship is affected by the presence of specific environmental factors (iodine intake, examination place and/or smoking) these environmental exposures were added in the regression analyses using a stepwise procedure, and the regression coefficients were compared. Moreover, interaction terms defined as the product of the genotype and the environmental exposure were added in the regression analyses and the significance of the interaction term was evaluated. Examination place was incorporated in the analyses as a dichotomized variable: Odense was coded as 0 and Copenhagen as 1. Smoking was also considered as a categorical variable: non-smokers coded as 0 vs. former or current smokers coded as 1. The measured urine iodine excretion was included as a continuous variable.

Quantitative genetic analyses

The proportion of variance of lnTSH accounted for by the variant of the TSHR-polymorphism was estimated as $Var(\beta_{TSHR}X_{TSHR})/Var(lnTSH)$, where $X_{TSHR}=1$ for carriers and 0 otherwise, and β_{TSHR} is the estimated regression coefficient for TSHR, controlling for age and sex.

Quantitative genetic analyses followed principles and methods as outlined elsewhere (23). Model fitting was done with Mx (24). In these analyses, the phenotypic variances are decomposed into genetic and environmental contributions (23). The genetic variance is further subdivided into an additive (A) component and a dominance (D) component. The environmental contribution is divided into a shared environmental component (C) and a unique (E) environmental component. The heritability is defined as the proportion of the total variance attributable to total additive genetic variance (23, 25). Series of univariate models were fitted in the five zygosity groups MZ males, DZ males, MZ females, DZ females and OS, separately. The ACE model was selected as a starting point of the modeling, and the significance of A, C, and E were tested by removing them sequentially in specific nested submodels.[23] Model fit was assessed using the –2 log likelihood (–2LL). To evaluate whether the (genotype) variation at the locus contributes to a significant fraction of the trait variation, models including and excluding the genotype (non-carriers versus carriers) as a covariate in the means model were compared, using hierarchical likelihood ratio chi-square tests. The difference between minus twice the log-likelihood for a reduced model (in which the effect of the genotype is set to zero) and that of the full model (including genotype information) is approximately chi-square distributed. The degrees of freedom (df) for this test are equal to the difference between the df for the reduced model and the full model. This gives a chi-square test with 1 df. In these analyses, the standardized genetic and environmental variance components estimates were assumed to be equal across gender. Being able to compare these hierarchical models, only twin pairs in which genotype information was available in both of the twins were included in these analyses. According to standard biometric practice (23), we assumed equal environ-

ment for MZ and DZ twins, no epistasis (gene-gene-interaction), and no gene-environment interaction or correlation.

RESULTS

Characteristics of the study population are given in table 1. The genotype distribution for the polymorphism was: Asp/Asp: 1053 (84.9%); Asp/Glu: 180 (14.5%) and Glu/Glu: 8 (0.6%). The allele frequencies did not deviate significantly from Hardy-Weinberg equilibrium. As evident from table 2, serum TSH levels decreased with the number of Glu727 alleles (1.78±0.93 vs. 1.61±0.85 vs. 1.53±0.75 mU/l). Carriers of the Glu727 allele had lower TSH levels (non-carriers vs. carriers: 1.78±0.93 vs. 1.60±0.84 mU/l, p=0.04). No significant differences were found for thyroid hormone levels, thyroid antibody levels or thyroid size. These findings were unchanged when excluding individuals with subclinical thyroid disease and/or positive thyroid antibody status (data not shown). With the exception of serum free T3 levels in smokers which were lower among carriers than non-carriers, neither stratification according to smoking status nor exclusion of individuals with ultrasonographically demonstrated thyroid nodules had a significant influence on the data (data not shown).

The results of the regression analyses are shown in table 3. Circulating serum TSH was significantly associated with the polymorphism, and the regression coefficient was not influenced by including iodine intake, examination place or tobacco smoking in the analyses (data not shown). A consistent genotype-phenotype relationship seemed to be the case. Moreover, no statistically significant evidence of interaction effects between the genotype and any of the environmental exposures was detected: (genotype × examination place inter-

Table 1: Characteristics of the study population.

Number of individuals	1241
Female/male ratio	623/618
Age (years)	36.7 ± 10.7
TSH mU/l	1.56 (1.03)
Free T4 (pmol/l)	12.82 ± 1.64
Free T3 (pmol/l)	6.19 ± 0.88
TPOab (kIU/l)	4.74 (3.07)
Tgab (kIU/l)	7.5 (4.99)
Non-smokers/smokers ratio	626/615
Examination place Odense/Copenhagen	692/549
Body mass index (kg/m^2)	24.5 ± 3.6
Thyroid size (ml) [a]	15.0 (6.4)

[a] n= 469

Twins with subclinical thyroid disease and positive thyroid antibody status are included. Values are means±SD or median (interquartile range) for variables with skewed distribution.

Table 2: The effect of the *TSHR*-Asp727Glu genotype on traits reflecting thyroid homeostasis.

Genotype	n	TSH mU/l	Free T4 pmol/l	Free T3 pmol/l	TPOab kIU/l	Tgab kIU/l	Size [a] ml
Asp/Asp	1053	1.78 (0.93)	12.85 (1.67)	6.21 (0.89)	64.7 (1298.4)	51.2 (1086.6)	16.1 (5.8)
Asp/Glu	180	1.61 (0.85)	12.65 (1.49)	6.11 (0.84)	15.4 (66.2)	15.3 (63.5)	14.9 (5.3)
Glu/Glu	8	1.53 (0.75)	12.81 (0.90)	6.23 (0.75)	34.7 (71.8)	6.8 (3.7)	14.8 (5.6)
Non-carriers	1053	1.78 (0.93) [b]	12.85 (1.67) [c]	6.21 (0.89) [c]	64.7 (1298.4) [c]	51.2 (1086.6) [c]	16.1 (5.8) [c]
Carriers	188	1.60 (0.84)	12.66 (1.47)	6.11 (0.83)	16.2 (66.3)	15.0 (62.1)	14.9 (5.3)

Results are presented as means ± SD

[a] In all, 469 individuals had an ultrasound scan to determine thyroid size. The genotype distribution was: Asp/Asp= 393; Asp/Glu=69; Glu/Glu=7.

[b] Non-carriers versus carriers: p=0.04

[c] Non-carriers versus carriers: non-significant

Table 3: Association between the Asp727Glu polymorphism in the TSH receptor gene and measures of thyroid homeostasis assessed by regression analysis.

Phenotype	n	β (95% CI)	p
lnTSH	1241	-0.14 (-0.24 - -0.04)	0.007
Free T4	1241	-0.18 (-0.42- 0.07)	0.158
Free T3	1241	-0.11 (-0.24- 0.02)	0.106
lnTPO	1241	-0.004 (-0.16- 0.15)	0.961
lnTgab	1240	0.02 (-0.09- 0.14)	0.694
lnThyroid size	469	-0.06 (-0.16- 0.03)	0.174

β signifies the regression coefficient of the polymorphism (non-carriers vs. carriers) with age and gender adjustment.

action, p = 0.966); (genotype × iodine excretion interaction, p = 0.926); (genotype × smoking interaction, p = 0.115).

Quantitative genetic modeling

As evident from table 4, the TSHR-Asp727Glu polymorphism accounted for only 0.91% of the total phenotypic variance in circulating serum TSH levels. For all phenotypes the best fitting model was an AE model. The inclusion of the Asp727Glu genotype as a covariate significantly improved the fit of the model (p=0.001), however the estimate for additive genetic effects without adjustment for the genotype was 68.2% (95% CI 62–73%), whereas inclusion of genotype status altered the estimate to 67.8 % (95% CI 61–73%). These results suggest that the Asp727Glu polymorphism accounts for a very small, but statistically significant, fraction of the total genetic variation in circulating serum TSH levels. The polymorphism accounted for 0.15-0.38% of the total phenotypic variance in thyroid hormone levels and thyroid size, and no significant differences on the point estimates of additive genetic influence were found. These results were unaltered when individuals with subclinical thyroid disease and/or positive thyroid antibody status were excluded (data not shown).

Table 4: Heritability estimates with and without adjustment for the TSHR-Asp727Glu polymorphism

Phenotype [a]	Number of individuals [b]	Variation explained (%)	Heritability estimate [c]	Heritability estimate [c] adjusted for TSHR-Asp727Glu polymorphism	p-value [d]
lnTSH	1220	0.91%	0.682 (0.62 - 0.73)	0.678 (0.61 - 0.73)	0.001
Free T4	1220	0.15%	0.599 (0.52 - 0.66)	0.598 (0.52 - 0.66)	0.215
Free T3	1220	0.19%	0.590 (0.51 - 0.66)	0.589 (0.51 - 0.66)	0.072
lnTPOab	1220	<0.01%	0.716 (0.66 - 0.76)	0.716 (0.66 - 0.76)	0.256
lnTgab	1220	0.01%	0.534 (0.43 - 0.62)	0.533 (0.43 - 0.62)	0.332
lnThyroid size	466	0.38%	0.802 (0.72 - 0.86)	0.800 (0.72 - 0.86)	0.252

[a] Twins with subclinical thyroid disease and positive thyroid antibody status are included.

[b] Only complete twin pairs are included in these analyses

[c] The proportion of the total variance attributable to total additive genetic variance. All heritability estimates are adjusted for age and gender

[d] Tested using a likelihood ratio chi-square test with 1 degrees of freedom. The difference between minus twice the log-likelihood for a reduced model (in which the effect of the genotype is set to zero) and that of the full model (including genotype information) is approximately chi-square distributed.

DISCUSSION

In the current study, we demonstrated that the Asp727Glu polymorphism in the *TSHR* gene is associated with circulating TSH levels in a healthy Danish population. Serum TSH decreased with the number of Glu727 alleles in a dose-dependent manner.

This replication of our initial findings (10), but in an independent and much larger study population clearly minimizes the risk of a false positive finding. On the other hand, our results demonstrate that the proportion of genetic influence explained by this particular polymorphism is small. Although the biological effect of the polymorphism is limited, the result is highly significant due to our large sample size. Thus, a substantial proportion of genetic variation, not accounted for by this *TSHR* gene polymorphism, still exists. This clearly suggests that multiple loci are involved in the control of serum TSH concentration (26). Such an organization may be regarded as an advantage for the overall control of thyroid homeostasis, which is then not as vulnerable to genetic mutations as if each polymorphism had a strong genetic impact. Thereby a more stable control is secured. Moreover, no associations with other measures of thyroid homeostasis were found. Circulating levels of thyroid hormones as well as thyroid size were all similar across the three genotype groups, perhaps due to the effect of the TSHR gene polymorphism on thyroid function being compensated by the associated change in serum TSH. Thus, the polymorphism may be regarded as a trait specific genetic influence.

The suggested association seems biologically plausible and functionally relevant. The fact that the TSHR-Glu727 allele in our study is associated with lower levels of serum TSH, but without effect on free T4 could imply a higher sensitivity of the variant versus the wild-type

TSHR, since less TSH is needed to produce normal free T4 levels. This hypothesis is supported by an *in vitro* study showing that the TSHR-Glu727 variant results in an exaggerated cAMP response of the receptor to TSH stimulation (13). However, others have not been able to replicate this finding (27, 28).

The TSH receptor gene is regarded as a thyroid specific candidate gene in the susceptibility to Graves' disease (29). Based on case-control association analyses, it has been found that the Asp727Glu polymorphism is more frequent in patients with Graves' disease than in healthy controls (30, 31). Other studies have been unable to replicate such an association (13, 30, 32-34). Moreover, in patients with Graves' disease the polymorphism does not seem to influence pre-treatment thyroid antibody levels (33). Neither was the polymorphism related with antibody levels in our healthy study population. The Asp727Glu polymorphism has also been found to be significantly more frequent in patients with toxic multinodular goiter than in normal individuals (13). Again, other studies have failed to demonstrate such an association (34, 35). Consequently, the polymorphism seems to have a weak influence on a broad variety of thyroid phenotypes (11). Whether the polymorphism exerts a weak primary disease-causing effect or is related to disease severity remains unclarified. In the current study, as well as in our previous studies, we have found that the Glu727 allele located in this thyroid specific gene contributes to decreasing TSH levels in healthy individuals. It is possible that the effect of this allele is related to thyroid function homeostasis in the clinical phenotypes as well - providing a link between the different thyroid dysfunction entities. In addition, circulating levels of TSH and thyroid hormones as well as thyroid size are of importance in the assessment and classification of thyroid dysfunction. Thus, investigation of these endophenotypes in the asymptomatic period may have the potential to improve our understanding of the development of thyroid disease (26).

A single, nominally significant association should be viewed as tentative until it has been independently replicated (36). In our study, the frequency of the Glu727 allele was comparable with those reported in other healthy populations, and using this large sample we were able to replicate our previous results (10) fulfilling the independent replication criterion. However, identification of this genetic polymorphism, accounting for a significant albeit very modest fraction of the total genetic variation in circulating TSH levels, should open the door for further genetic analyses, because unmeasured confounders – that is other associated genetic loci and/or environmental exposures - may be of importance (36). Our study population is recruited from a single European geographic area, and we expect that population stratification effects are of limited importance.

To further characterize the genotype phenotype relationship, we investigated the possibility of interaction between the Asp727Glu polymorphism and relevant environmental exposures. Yet, studying the interplay between measured genetic and environmental factors may require larger sample sizes; and with a simple interaction model we were unable to detect that the effect of the polymorphism was modified by iodine intake (measured as iodine

excretion) or cigarette smoking. In these analyses, the variable "examination place" served as a surrogate for iodine intake. This allowed us to use the whole study population. However, the results were consistent using the actually measured urinary iodine excretion levels.

To conclude, our results confirm previous data showing that the TSHR-727Glu variant is associated with lower plasma TSH levels and extend them by showing the proportion of genetic variation accounted for by this *TSHR* gene polymorphism is small. The polymorphism was not associated with circulating thyroid hormone levels, thyroid antibody levels or thyroid size.

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Chapter 7

Effects of serum TSH and FT4 levels and the TSHR-Asp727Glu polymorphism on bone: the Rotterdam Study

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ABSTRACT

Introduction: TSH and thyroid hormone may have independent effects on bone. In this study we investigated the association of TSH and FT4 with different bone parameters in humans. TSH and FT4 are known to be associated with BMI and a higher BMI gives a higher bone mineral density (BMD). Thus, we analyzed if the effects of TSH and FT4 on bone are mediated by BMI. Since TSH exerts its biological effect through the TSH receptor (TSHR), the TSHR gene might be a candidate gene affecting bone mass. The TSHR-Asp727Glu polymorphism is associated with lower TSH levels. Therefore, we examined the association of this polymorphism with bone parameters.

Methods: Genotypes were determined by Taqman assay in 4934 elderly Caucasian men and women of the Rotterdam Study, of whom BMD and bone geometry data were available. In a random set of 1327 subjects serum thyroid parameters were available.

Results: Femoral neck BMD as well as narrow neck BMD and cortical thickness increased with serum TSH. However, FT4 was more strongly and negatively associated with bone parameters. Regression models showed BMI-dependent and -independent effects of both TSH and FT4 on bone. Carriers of the TSHR-Glu^{727} allele had a 2.3% higher femoral neck BMD.

Conclusion: In line with the effect of TSH on bone in mice, serum TSH shows a positive trend with BMD in humans, a finding which is strengthened by the association between the TSHR-Asp727Glu polymorphism and femoral neck BMD. However, serum FT4 has a much greater influence on bone than TSH.

INTRODUCTION

Thyroid hormone plays an important role in skeletal growth and bone homeostasis. Children with hypothyroidism show delayed bone maturation and lower height (1), whereas in adults excessive thyroid hormone levels are associated with bone loss and a reduction in bone mineral density (BMD) at multiple skeletal sites (2). Studies in patients with subclinical hyperthyroidism show that minor changes in thyroid hormone and/or thyroid stimulating hormone (TSH) levels can already have important consequences for BMD (3).

Since thyroid hormone is necessary for normal bone function, mechanisms of thyroid hormone action on bone have been studied extensively (4, 5). In addition to effects of thyroid hormone on the skeleton, TSH has recently been shown to have a direct effect on bone (6, 7). TSHR knockout mice die by 10 weeks of age and display high-turnover osteoporosis, which supplementation with thyroid hormone fails to reverse. Heterozygous $TSHR^{+/-}$ mice thrive normally, but still have pronounced osteoporosis. TSH inhibits through the TSHR both osteoclastic bone resorption and osteoblastic bone formation. In this study we analyzed whether serum TSH and free T4 (FT4) levels are associated with bone parameters in a large and homogeneous population-based sample of Caucasian elderly men and women.

While bone mass is also determined by environmental factors such as physical activity and smoking, genetic factors have been estimated to determine up to 80% of the variation in BMD (8-10). The heritable component is mediated by multiple genes, each having a small, but cumulative effect. Since TSH exerts its biological effect through the TSHR, we analyzed the TSHR gene as a possible candidate gene impacting on BMD and fracture risk. We studied a common polymorphism in the TSHR gene, i.e. the TSHR-Asp727Glu polymorphism. The TSHR-Glu^{727} variant is associated with lower serum TSH but not with FT4 levels in several Caucasian populations (11, 12), which suggests a higher activity of the Glu^{727} variant versus the Asp^{727} form of the receptor.

It is difficult to discriminate direct effects of TSH from those of thyroid hormones on peripheral tissues such as bone. In an attempt to make this distinction, we studied the TSHR-Asp727Glu polymorphism, since this polymorphism has been found to be associated with only serum TSH levels, and not with other serum thyroid parameters. Potential effects of this polymorphism on bone parameters are therefore likely to be direct effects of TSH.

SUBJECTS AND METHODS

Study population

The Rotterdam Study is a population-based, prospective cohort study of men and women, aged 55 years and over. The study was designed to investigate the incidence and determinants of chronic disabling diseases in an aging population (13) The Rotterdam Study was

approved by the Medical Ethics Committee of Erasmus University Medical School and written informed consent was obtained from 7806 subjects.

Subjects

Association analyses between serum thyroid parameters, the TSHR-Asp727Glu polymorphism and bone parameters were first performed in a randomly selected subgroup of 1327 subjects of the Rotterdam Study, in which serum TSH, FT4 and antibodies to thyroid peroxidase (TPO-Abs) were available. Secondly, analyses were performed in 4934 subjects for whom complete data on bone parameters were available. The subjects, in which serum thyroid parameters and TPO-Abs were available, are also part of this group.

Plasma analyses

TSH levels were measured with TSH Lumitest (Henning, Berlin, Germany). Serum FT4 was measured by a chemoluminescence assay (Vitros, ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK). TPO-Abs were assessed by ELISA (Milenia, DPC, Los Angeles, USA).

Measurement of bone parameters and anthropometry

BMD (expressed in grams per square centimeter) was measured at the femoral neck and the lumbar spine (vertebrae L2-L4) by dual energy x-ray absorptiometry (DXA; Lunar DPX-L densitometer, Lunar Corp., Madison, WI). The hip structural analysis (HSA) software developed by Thomas J. Beck (14) was used to measure hip bone geometry and structure parameters from the DXA scans of the narrow-neck region across the narrowest point of the femoral neck, as described previously (15).

Anthropometric measurements were obtained at the research center. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared.

Fracture follow-up

Both at baseline (1990-1993) and at the third follow-up visit thoracolumbar radiographs of the spine were obtained. The presence of vertebral fractures was assessed by the McCloskey/Kanis method and analyzed as described previously (16, 17). The follow-up radiographs were available for 2699 individuals, who survived after an average follow-up period of 8.7 years after baseline center visit and who were still able to come to the research center. The non-vertebral fracture analysis is based on follow-up data collected from baseline until January 1st, 2002, comprising an average follow-up period of 8.1 years. Fracture events were retrieved by research physicians as described previously (15).

Genotyping

Participants were genotyped for the Asp727Glu polymorphism (rs1991517) (11) of the TSHR gene. We refrained from analyzing other polymorphisms in the TSHR gene, such as TSHR-Pro52Thr and TSHR-Asp36His, since these either have a low frequency or have not been associated with serum thyroid parameters in vivo to date (11). Genotypes were determined in 2 ng genomic DNA using the Taqman allelic discrimination assay (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Reactions were performed on the Taqman Prism 7900HT in 384 wells format in 2 µl reaction volume.

Statistical analysis

In the subgroup, in which serum thyroid parameters were available, analyses were carried out after exclusion of subjects on thyroid medication and/or amiodarone, with clinical hypo- or hyperthyroidism (FT4 <11.0 or >25.0 pmol/L) and with TPO-Abs >35 IU/L (n=176). Due to a non-normal distribution, TSH was logarithmically transformed and relations between variables were assessed using linear regression, adjusted for age, gender and BMI. For the TSHR-Asp727Glu polymorphism, deviation from Hardy-Weinberg equilibrium proportions was analyzed using a Chi-square test. Genotype-related differences were adjusted for age, gender and BMI and tested by analysis of covariance (ANCOVA). Fisher's LSD correction for multiple testing was used.

In the complete cohort, association of the TSHR-Asp727Glu polymorphism with bone parameters was tested after exclusion of subjects with thyroid pathology, leaving 4801 subjects eligible for analysis.

To estimate non-vertebral fracture risk, we used Cox proportional hazard models, taking potential differences in follow-up time into account. The relative risk and 95% confidence interval of vertebral fractures were calculated by a logistic regression model.

Spearman's correlation coefficient was used to assess the association between serum TSH and FT4. P values are two-sided throughout and were considered significant if $P < 0.05$. Data were analyzed using SPSS 10.0.7 for Windows (SPSS, Inc., Chicago, IL).

RESULTS

Serum TSH and FT4 and bone parameters

Baseline characteristics of the subgroup, in which serum thyroid parameters were available, and the complete study cohort are shown in Table 1.

Table 2 shows the associations between serum TSH and bone parameters. Femoral neck BMD increased with serum TSH level (P=0.06). Also femoral narrow neck BMD (P=0.01) and cortical thickness (P=0.01) increased with serum TSH concentrations (Table 2). Since serum TSH levels are positively associated with BMI (18, 19) and obese subjects have a higher BMD

(9, 20), we tested whether the association between serum TSH levels and BMD was (partly) driven by the positive relation with BMI. Serum TSH was positively associated with BMI (β_{lnTSH}=0.34, p=0.02) and we observed a close association of BMI and femoral neck BMD (data not shown). So, in addition to age and gender, we adjusted for BMI in the analysis of serum TSH and bone parameters. The same trends were observed, but the associations became borderline significant (Table 2). We also analyzed covariance of the different bone parameters with both serum TSH and BMI in a regression model, together with an interaction term (serum TSH x BMI). This model showed no evidence of interaction, but suggested independent and additive effects of serum TSH and BMI on bone parameters.

Table 3 shows the associations between serum FT4 and bone parameters. Serum FT4 was significantly and consistently associated with all bone parameters, except narrow neck width. For instance, femoral neck BMD and cortical thickness decreased with increasing FT4. In the regression model when BMI was added as a covariate, FT4 remained significantly associated with bone parameters.

Serum TSH and FT4 are tightly coupled in the pituitary-thyroid axis (2). In this population, lnTSH and FT4 were also correlated with a correlation coefficient of –0.206 (P<0.001) (Fig. 1). Since both serum TSH and FT4 were associated with bone parameters, we included both variables in a multivariate regression model. Only serum FT4 remained significantly associated with bone parameters in this model (data not shown).

When we performed gender-specific analyses of the association of serum TSH and FT4 with bone parameters, similar trends were observed. To maximize power, we combined men

Table 1: Baseline characteristics of the study populations

	Subgroup with serum thyroid parameters	Complete Study Cohort	P
N	1151	4801	
Age (yrs)	68.72 ± 7.54	67.65 ± 7.78	<0.001[a]
Gender (M/F)	479 / 672	2101 / 2700	0.19[a]
BMI (kg/m^2)	26.30 ± 3.63	26.26 ± 3.55	0.99[b]
Femoral Neck BMD (g/cm^2)	0.86 ± 0.14	0.87 ± 0.14	0.98[b]
Narrow Neck BMD (g/cm^2)	0.68 ± 0.14	0.70 ± 0.14	0.09[b]
Narrow Neck Width (cm)	3.21 ± 0.33	3.21 ± 0.32	0.23[b]
Cross Sectional Area (cm^2)	2.09 ± 0.49	2.14 ± 0.49	0.27[b]
Average Cortical Thickness (cm)	0.130 ± 0.027	0.133 ± 0.028	0.09[b]
Average Buckling Ratio	14.25 ± 4.23	13.88 ± 3.93	0.17[b]
Lumbar Spine BMD (g/cm^2)	1.09 ± 0.20	1.09 ± 0.20	0.72[b]
TSH (mU/L)	1.48 [0.98-2.23]		
FT4 (pmol/L)	16.34 ± 2.68		

Data are shown as mean ± SD or as median [IQR].

[a] Chi-square for gender, Anova for age

[b] Anova, adjusted for age and gender

Table 2: Linear regression coefficients (ß) for the relationships of serum lnTSH with bone parameters

	Adjusted for age and gender		Adjusted for age, gender and BMI	
	ß (95%CI)	P	ß (95%CI)	P
BMI	0.343 (0.068-0.618)	0.02		
Lumbar spine BMD	0.009 (-0.006-0.023)	0.24	0.004(-0.010-0.018)	0.59
Femoral Neck BMD	0.009 (0.000-0.019)	0.06	0.006 (-0.003-0.015)	0.20
Narrow Neck BMD	0.014 (0.003-0.024)	0.01	0.009 (-0.001-0.019)	0.07
Cross Sectional Area	0.039 (0.006-0.073)	0.02	0.026 (-0.005-0.057)	0.10
Narrow Neck Width	-0.007 (-0.028-0.014)	0.53	-0.007 (-0.028-0.014)	0.51
Cortical Thickness	0.003 (0.001-0.005)	0.01	0.002 (0.000-0.004)	0.07
Buckling Ratio	-0.297 (-0.531-0.145)	0.10	-0.193 (-0.531-0.145)	0.26

Table 3: Linear regression coefficients (ß) for the relationships of serum FT4 with bone parameters

	Adjusted for age and gender		Adjusted for age, gender and BMI	
	ß (95%CI)	P	ß (95%CI)	P
BMI	-0.093 (-0.170--0.015)	0.02		
Lumbar spine BMD	-0.005 (-0.010--0.001)	0.009	-0.004(-0.008-0.000)	0.04
Femoral Neck BMD	-0.003 (-0.006--0.001)	0.01	-0.003 (-0.005-0.000)	0.05
Narrow Neck BMD	-0.005 (-0.008--0.002)	0.001	-0.004 (-0.007--0.001)	0.004
Cross Sectional Area	-0.019 (-0.028--0.010)	<0.001	-0.016 (-0.024--0.007)	<0.001
Narrow Neck Width	-0.004 (-0.010-0.002)	0.19	-0.004(-0.010-0.002)	0.20
Cortical Thickness	-0.001 (-0.002-0.000)	0.001	-0.001 (-0.001-0.000)	0.005
Buckling Ratio	0.127 (0.028-0.225)	0.01	0.098 (0.003-0.193)	0.04

and women in the analyses. Since subjects of the Rotterdam Study were all aged 55 years or older, we were not able to analyze women according to their menopausal status. However, additional adjustment for age at menopause did not essentially change the observed associations.

The TSHR-Asp727Glu polymorphism and bone parameters

DNA from participants of the Rotterdam Study was genotyped for the TSHR-Asp727Glu polymorphism. In the subgroup, DNA was available from 1089 subjects for genotyping. The genotyping success rate was 98.1%, with 21 samples that failed genotyping. Genotype frequencies of the TSHR-Asp727Glu polymorphism (Asp/Asp = 947 (88.7%), Asp/Glu = 119 (11.1%) and Glu/Glu = 2 (0.2%) did not deviate from Hardy Weinberg equilibrium proportions. The Glu^{727} allele had a frequency of 5.8 %, which is similar to previous studies in Caucasians (11). As only few homozygous subjects were identified for the polymorphism, we combined heterozygotes and homozygotes into variant carriers. Carriers of the TSHR-Glu^{727} variant had a 2.3% higher femoral neck BMD (P=0.03) and had a 12.6% lower TSH level (P=0.04) (Fig. 2, Table 4). In agreement with previous studies, the TSHR-Asp727Glu polymorphism was not associated with serum FT4 levels. Since Glu^{727} carriers showed a trend towards lower TSH

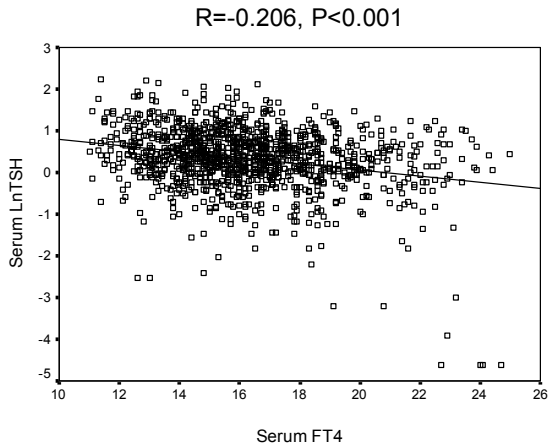

Figure 1: Scatterplot between serum lnTSH and FT4.

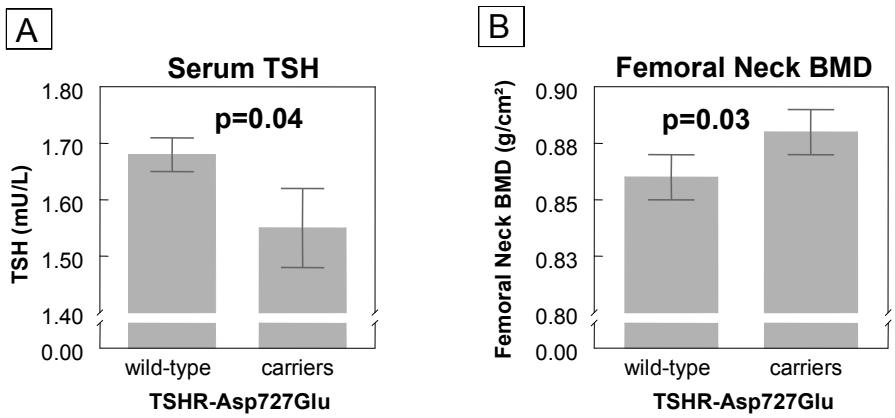

Figure 2: Differences in serum TSH levels (A) and femoral neck BMD (B) between wild type and carriers of the TSHR-Glu^{727}allele in the subgroup, in which serum thyroid parameters were available. The data were adjusted for age, gender and BMI.

levels, this might mask the associations between the TSHR-Asp727Glu polymorphism and bone parameters. For this reason, TSH was added as a covariate in the analysis of the polymorphism with bone parameters. The results essentially remained the same (Table 4).

In the complete study cohort the genotype frequencies of the TSHR-Asp727Glu polymorphism (Asp/Asp = 4170, Asp/Glu = 600 and Glu/Glu = 31) were also consistent with Hardy-Weinberg equilibrium proportions. In this group similar results were found for femoral

Table 4: Bone parameters by TSHR-Asp727Glu genotype

	TSHR-Asp727Glu subgroup				TSHR-Asp727Glu complete study cohort		
	Asp/Asp	Asp/Glu + Glu/Glu	P	P_{TSH}	Asp/Asp	Asp/Glu + Glu/Glu	P
N	947	121			4170	631	
BMI	26.27 ± 0.12	26.53 ± 0.33	0.44	0.37	26.29 ± 0.05	26.06 ± 0.14	0.12
Lumbar spine BMD	1.09 ± 0.01	1.10 ± 0.02	0.42	0.40	1.09 ± 0.01	1.10 ± 0.01	0.50
Femoral Neck BMD	0.86 ± 0.01	0.88 ± 0.01	0.03	0.02	0.87 ± 0.01	0.88 ± 0.01	0.01
TSH	1.68 ± 0.03	1.55 ± 0.07	0.04				
FT4	16.35 ± 0.09	16.38 ± 0.24	0.89				
N	748	100	P	P_{TSH}	3372	520	P
Narrow Neck BMD	0.68 ± 0.01	0.70 ± 0.01	0.16	0.12	0.70 ± 0.01	0.71 ± 0.01	0.16
Narrow Neck Width	3.21 ± 0.01	3.20 ± 0.02	0.63	0.60	3.21 ± 0.01	3.21 ± 0.01	0.83
Cross sectional Area	2.09 ± 0.01	2.13 ± 0.04	0.32	0.26	2.14 ± 0.01	2.16 ± 0.02	0.15
Cortical Thickness	0.129 ± 0.001	0.133 ± 0.002	0.13	0.10	0.133 ± 0.001	0.134 ± 0.001	0.15
Buckling Ratio	14.29 ± 0.15	14.05 ± 0.40	0.58	0.52	13.90 ± 0.06	13.72 ± 0.16	0.27

Data are shown as mean ± SEM. Logarithmic transformations were used for serum TSH levels. All data were adjusted for age, gender and BMI. P_{TSH} represents the P-value, when additionally corrected for serum TSH.

neck BMD: carriers of the Glu^{727} allele had a higher femoral neck BMD than non-carriers. In addition, the TSHR-Asp727Glu polymorphism was in a dose-dependent manner associated with higher femoral neck BMD (Asp/Asp: 0.87 ± 0.01; Asp/Glu: 0.88 ± 0.01; Glu/Glu: 0.89 ± 0.01 g/cm^2, P_{tr}=0.009).

Both in the subgroup and in the complete cohort, the TSHR-Asp727Glu polymorphism was not associated with BMI.

The TSHR-Asp727Glu polymorphism and fracture risk

We analyzed the risk for vertebral and non-vertebral fractures by the TSHR-Asp727Glu polymorphism in the subgroup in which serum thyroid parameters were available and in the complete study cohort, but no associations were found. Also, serum TSH and FT4 were not associated with fracture risk (data not shown).

DISCUSSION

In this study we examined whether serum TSH and FT4 levels and a polymorphism in the TSHR gene have an impact on BMD, bone geometry and the risk of osteoporotic fracture. We showed in a population of elderly Caucasians that femoral neck BMD as well as femoral narrow neck BMD and cortical thickness increased with serum TSH. Furthermore, serum FT4 was significantly and consistently associated with bone parameters in a negative manner. The TSHR-Glu^{727} allele was associated with a higher BMD at the femoral neck and lower serum

TSH, but not FT4 levels. No association of the TSHR polymorphism or serum TSH or FT4 with fracture risk was found.

Only a few studies have been published on the expression of TSHR in human bone cells or the influence of serum TSH on bone in humans. Tsai et al. found weak evidence for the presence of the TSHR in primary cultures of human osteoblast-like cells (21). On the other hand, a recent study conducted by Morimura et al. (22) demonstrated the presence of TSHR mRNA in human osteoblast cell lines. Recently, Mazziotti et al. showed that an increase in serum TSH levels accomplished by short-term recombinant human TSH stimulation, is accompanied by a reversible inhibition of bone resorption (23). Moreover, in a study of more than 600 postmenopausal women, Bauer et al. showed that women who have low serum TSH levels, have an increased risk for hip and vertebral fractures (24). In this study, low serum TSH was also negatively associated with femoral neck BMD, narrow neck BMD and cortical thickness. These results confirm the findings of Mazziotti et al. and suggest direct effects of TSH on bone in humans, similar to those recently demonstrated in a study of TSHR KO mice (7). We did not find an association of serum TSH with lumbar spine BMD. This might be due to the fact that the BMD measurements of the lumbar spine are influenced by osteoarthritis and therefore cannot be accurately assessed (25).

Recent studies have shown that serum TSH levels are positively associated with BMI and the occurrence of obesity (18, 19). In our study serum TSH also showed a positive association with BMI. Since obese subjects have a higher BMD (9, 20), we tested whether the association between serum TSH levels and BMD is explained by the positive relation with BMI. The effect of serum TSH on bone seems partially mediated through BMI and partly through a direct effect of TSH itself, since the regression models suggested independent and additive effects of serum TSH and BMI on bone parameters.

The associations between serum TSH and bone parameters are consistent with a direct effect of TSH. However, these findings could also result from altered levels of thyroid hormone, since TSH and FT4 are tightly coupled in the pituitary-thyroid axis (2). In this population, serum FT4 showed a strong negative association with several bone parameters pointing to an important role of thyroid hormone itself on bone function. Considering the strength of the associations of serum TSH and FT4 with bone parameters, FT4 appears to have a greater impact on bone development and turnover than any direct effect of TSH. This notion is strengthened by the observation that only serum FT4 remains significantly associated with bone parameters in the multivariate regression model including both serum TSH and FT4, besides the other co-variables age, gender and BMI. This is in accordance with studies described in a recent paper by Bassett et al (26), who conclude that FT4 excess rather than TSH deficiency explains the detrimental effects of hyperthyroidism on bone.

In an attempt to distinguish between direct effects of TSH on bone and those mediated by FT4 we studied the effect of the Asp727Glu polymorphism in the TSHR gene. This polymorphism is associated with relatively low TSH levels, but normal FT4 levels (11, 12), a finding we

were able to replicate in this study population, but also in another independent population of more than a thousand subjects (unpublished data by Van der Deure, Peeters and Visser). This would suggest a higher activity of the receptor in carriers of the Glu^{727} allele, since less TSH is needed to produce normal FT4 levels. There is indeed one in vitro study showing that the TSHR-Glu^{727} variant results in an increased cAMP response of the receptor to TSH (27); however, others have not been able to replicate this (28, 29), which may suggest that the Asp727Glu polymorphism is linked to another functional polymorphism elsewhere in the TSHR gene.

The TSHR-Glu^{727} allele was associated with a higher femoral neck BMD and showed a trend towards thicker cortices. These data support the finding of a direct effect of TSH on bone as previously observed in mice (7). When we additionally added serum TSH as a covariate in our model, the estimated means for the TSHR-Asp727Glu genotypes of the different bone parameters remained essentially the same; however, the associations became slightly stronger, suggesting that the effect of the Glu^{727} variant on bone was partially masked by the compensatory decrease in serum TSH levels. Although the TSHR-Glu^{727} variant was associated with higher bone mass, we did not observe an increased fracture risk in subjects with the wild-type TSHR versus the variant carriers. In this respect, it should be noted that we could have detected a relative risk for non-vertebral fractures of 1.5 with 80% power at a 5% type I error probability in this study. However, we cannot exclude a relative risk of 1.5 or less. A larger study population would be required to demonstrate such an effect.

Since no increased fracture risk was observed and the difference in femoral neck BMD between carriers and non-carriers of TSHR-Asp727Glu polymorphism was small, one could wonder if this polymorphism is clinically relevant. However, it is important to realize that people carry this polymorphism for life. Since this polymorphism is associated with altered serum TSH levels, this might, for instance, point to a lifelong altered thyroid function set point. Furthermore, new insights can be obtained about the physiological role of thyroid pathway genes. Our finding that the TSHR-Asp727Glu polymorphism is associated with femoral neck BMD is in support of a direct albeit modest effect of serum TSH on bone.

This study finds associations between serum TSH and FT4 levels and a polymorphism in the TSHR gene with bone parameters. The results suggest that the TSHR-Asp727Glu polymorphism is associated with higher bone mass and that increased serum TSH levels give rise to less fragile bone in humans via BMI-dependent and independent pathways. However, thyroid hormone itself has a much larger effect on bone, suggesting that the negative effect of (subclinical) hyperthyroidism on bone is more likely to be the effect of high FT4 instead of low TSH. Further studies are needed to confirm our findings and to elucidate the exact mechanisms by which both serum TSH, FT4 and the polymorphism in the TSHR affect bone parameters.

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Chapter 8

The effect of genetic variation in the type 1 deiodinase gene on the inter-individual variation in serum thyroid hormone levels. An investigation in healthy Danish twins.

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ABSTRACT

Introduction: Genetic factors have a considerable influence on serum thyroid hormone levels. The C785T and A1814G polymorphisms, located in the 3' untranslated region of the type 1 deiodinase (D1) gene have been associated with serum FT4 and rT3 levels.

Objective: In healthy Danish twins, we examined the association of these polymorphisms with serum thyroid hormone levels and determined the proportion of genetic influence explained by these variants. We analyzed the underlying functional mechanism by performing mRNA stability measurements and analyzed the effect of these variants on D1 activity.

Methods: Serum thyroid measurements and genotypes of the D1-C785T and D1-A1814G polymorphisms were determined in 1192 twins. Structural equation modeling was used to determine heritability estimates. Functional analyses were carried out in D1-transfected JEG3 cells.

Results: Carriers of the D1-785T allele had 3.8% higher FT4 and 14.3 % higher rT3 levels, resulting in a lower T3/T4 and T3/rT3 ratio and a higher rT3/T4 ratio. This polymorphism explained 0.87% and 1.79%, respectively, of the variation in serum FT4 and rT3. The D1-A1814G polymorphism was not associated with serum thyroid hormone levels. No differences in D1 mRNA decay rate or D1 activity were observed between wild-type D1 and the 2 variants.

Conclusion: The D1-C785T polymorphism is consistently and significantly associated with serum thyroid hormone levels. However, the proportion of genetic influence explained by this particular polymorphism is small. No effect of the polymorphism on D1 mRNA decay rate or D1 activity was observed. The underlying functional mechanism needs to be elucidated.

INTRODUCTION

The thyroid gland produces predominantly the pro-hormone thyroxine (T4), which has to be deiodinated to 3,3'5-triiodo-L-thyronine (T3) to become biologically active. The conversion of thyroid hormones is regulated by three different iodothyronine deiodinases (D1-D3) (1). The tissue distribution and enzymatic activity of the deiodinases differ and they all play a distinct physiological role. For instance, D1 is highly expressed in liver and kidney and plays an important role in the production of T3 from T4 and in the clearance of reverse T3 (rT3).

Polymorphisms in D1 have been associated with serum thyroid hormone levels (2): the D1-C785T polymorphism in the 3' untranslated region (3'UTR) is associated with higher rT3 levels and a lower T3/rT3 ratio, pointing towards a lower D1 activity in carriers of the T allele (2, 3). Another polymorphism in the 3'UTR of D1, D1-A1814G, is associated with a higher T3/rT3 ratio, suggesting that the D1-1814G variant might result in increased D1 activity (2, 3). Recently, these findings have been replicated in a large population of elderly Caucasians of the Rotterdam Scan Study (4). To date only 1 polymorphism in D2 and no polymorphisms in D3 have been associated with serum thyroid hormone levels (3). The D2-ORFa-Gly3Asp was associated with serum thyroid parameters in a population of 156 healthy blood donors, but this could not be replicated in a population of approximately 350 elderly men, nor in a cohort of more than 1000 elderly Caucasians (4, 5).

Genetic factors exert considerable influence on most of the thyroid function measurements, with heritability estimates ranging between 23-65% (6, 7). Recently, we have determined the contribution of the thyrotropin (TSH) receptor Asp727Glu polymorphism to the genetic variance in serum TSH levels (8). In this study, we examined the association of the D1-C785T and D1-A1814G polymorphisms with thyroid function measurements in healthy Danish twins and determined the contribution of the polymorphisms to the overall variance in serum thyroid hormone levels. To analyze the underlying functional mechanism, we determined mRNA degradation rates, as these polymorphisms in the 3'UTR might alter D1 mRNA stability (9). In addition, we studied the effect of these variants on D1 activity by analysis of rT3 deiodination in JEG3 cells transfected with wild-type or variant D1.

MATERIALS AND METHODS

Study population

The present study is part of a nationwide project (GEMINAKAR) investigating the relative influence of genetic and environmental factors on various traits related to the metabolic syndrome and cardiovascular risk factors. Based on a questionnaire survey concerning physical health and health related behavior performed in 1994, a representative sample of self-reported healthy twin pairs was recruited from the population-based Danish Twin Registry

(10). A detailed description of the ascertainment procedure can be found elsewhere (6, 11, 12). In the GEMINAKAR study 1512 individuals (756 twin pairs) were examined. Blood samples for thyroid measurements and genotype information were available in 1266 individuals, distributed in 554 monozygotic (MZ), 474 same sex dizygotic (DZ) and 238 opposite sex (OS) twin individuals. In the MZ twin pairs, in which only one of the twins was genotyped, we assumed identical genotypes. Zygosity was established by analysis of nine highly polymorphic restriction fragment length polymorphisms and microsatellite markers widely scattered through the genome with an Applied Biosystems AmpFISTR Profiles Plus kit, according to the manufacturer's instructions (Perkin-Elmer Co. Foster City, CA, USA).

Twin pairs in which one or both twins had self-reported thyroid disease (22 twin pairs) or biochemical thyroid disease (15 twin pairs) were excluded, leaving 1192 (524 MZ, 442 DZ and 226 OS twin individuals). Due to missing genotype data, the D1-A1814G polymorphism was only established in 1076 individuals.

Written informed consent was obtained from all participants and the study was approved by all regional Danish Scientific-Ethical Committees (case file 97/25 PMC).

Serum analyses

Serum TSH was measured using a time-resolved fluoroimmunometric assay (AutoDELFIA hTSH Ultra Kit, Perkin Elmer/Wallac, Turku, Finland). Serum free T4 (FT4) and serum free T3 (FT3) were determined using the AutoDELFIA FT4 and FT3 kits (Perkin Elmer/Wallac, Turku, Finland). Twin pairs were analyzed within the same run. Total T4 (T4), total T3 (T3) and reverse rT3 (rT3) were measured with in-house RIA's (6, 13).

Genotyping

DNA was isolated from peripheral leukocytes according to standard procedures. The D1-C785T (rs11206244) and D1-A1814G (rs12095080) polymorphisms were determined by 5'fluorogenic Taqman assays. For both polymorphisms, we used Assays-by-Design (www.appliedbiosystems.com). PCR cycling reactions were performed in 384 wells format in a 2 µl reaction volume on an ABI 9700 PCR system (Applied Biosystems Inc., Foster City, CA, USA). Results were analyzed by the ABI Taqman Prism 7900HT using the sequence detection system 2.22 software (Applied Biosystems).

Plasmids and construction of D1-C785T and D1-A1814G variant

Human D1 inserted in the pCDM plasmid (pCDM-hD1) was kindly provided by Drs Antonio C. Bianco and Ann Marie Zavacki (Brigham and Women's Hospital, Harvard Institutes of Medicine, Boston, MA). This clone was shuttled into pcDNA3 (Invitrogen, Breda, The Netherlands) using *Hind*III and *Not*I restriction sites.

The C785T and A1814G polymorphisms were introduced in the D1 cDNA as follows: a PCR reaction was performed on DNA of subjects wild-type or homozygous variant for either one

of the polymorphisms with forward primer GGAATGCCTGATTCGTTCTC and AGATCACACG-GAAGCCATC as reverse primer, generating a PCR-product of 1207 bp. This PCR-fragment was exchanged with part of the D1 cDNA in pcDNA3 via *Xcm*I en *Not*I. The constructs were fully sequenced on an automated ABI 3100 capillary sequencer, using the Big Dye terminator cycle sequencing method (Applied Biosystems). These plasmids are devoid of regulatory elements involved in the regulation of D1 expression by cell- and tissue-specific factors. pcDNA3-hMCT8 was constructed as previously described (14).

Cell culture

JEG3 cells were cultured in 6 or 24-well dishes (Corning, Schiphol, The Netherlands) with DMEM/F12 medium (Invitrogen), containing 9% heat-inactivated fetal bovine serum (Invitrogen) and 100 nM sodium selenite (Sigma, Zwijndrecht, The Netherlands).

mRNA stability assays

JEG3 cells were cultured in 6-well dishes and transfected with 500 ng wild-type or variant D1. After 48 h culturing, cells were put on fresh medium with 7.5 µl/ml actinomycin D (Sigma). Cells were harvested 0, 8 and 24 h after addition of actinomycin D. Subsequently, cells were washed with PBS and RNA was isolated using the high pure RNA isolation kit (Roche Diagnostics, Almere, The Netherlands). For cDNA synthesis 500 ng RNA and TaqMan RT reagents (Roche Diagnostics) were used in a total volume of 50 µl.

Two µl cDNA was used for real-time PCR on the ABI Prism 7700 sequence detection system (Applied Biosystems). Real-time PCRs were performed as described previously (15).

rT3 metabolism assay

[^{125}I]rT3 was purchased from Perkin Elmer (Boston, USA) and unlabeled rT3 from Henning GmbH (Berlin, Germany). FuGENE6 transfection reagent was obtained from Roche Diagnostics (Indianapolis, USA).

JEG3 cells were cultured in 24-well dishes and transfected with 1) 100 ng pcDNA3-hMCT8 and 100 ng empty vector, or 2) 100 ng pcDNA3-D1, D1-785T or D1-1814G plus 100 ng pcDNA3-MCT8. After 48 h culturing, cells were incubated for 4 h at 37 C with 1 nM [^{125}I]rT3 in 0.25 ml Dulbecco's PBS with 0.1% BSA and 0.1% glucose. After incubation, medium was harvested and analyzed by HPLC as described previously (14).

Statistical analysis

Data were analyzed using STATA statistical software (STATA Corporation, College Station, TX) and SPSS 10.1.0 for Windows (SPSS inc., Chicago, IL). P values are two-sided throughout and were considered significant if $P < 0.05$. If not stated otherwise, results are displayed as mean ± SD. For the polymorphisms, deviation from Hardy-Weinberg equilibrium proportions was analyzed using a Chi-square test. The program PHASE was used for reconstructing haplotypes

from the genotype data (16). The haplotype alleles were defined as "C-A" representing a C nucleotide for the C785T polymorphism and an A nucleotide for the A1814G polymorphism. Haplotype alleles were coded as haplotype numbers 1 through 4 in order of decreasing frequency in the population.

Associations between the polymorphisms, haplotypes and serum thyroid hormone levels were assessed using regression analysis. The statistical inference measures (i.e, p-values and CI) were computed using a technique (the cluster option in STATA) that takes the dependency of twin data into account. The genotype or haplotype information was incorporated in the regression analyses by coding the non-carriers as 0, heterozygous carriers as 1 and homozygote carriers as 2. Due to non-normal distribution of most of the serum thyroid hormone levels, these were transformed by the natural logarithm, and adjustment for age and gender was performed.

In the case of significant associations between polymorphisms and serum thyroid variables, the proportion of the total phenotypic variance was determined as described previously (8). Furthermore, the effect of the polymorphisms on the heritability estimates was quantified by incorporating the genotype information into structural equation modeling using the Mx program (17, 18). To evaluate whether the (genotype) variation at the locus contributes to a significant fraction of the genetic variation, models including and excluding the genotype (non-carriers versus carriers) as a covariate in the means model were compared, using hierarchical likelihood ratio chi-square tests (8).

For the *in vitro* experiments, Mann-Whitney U-tests were used to test whether there were statistically significant differences between wild-type and variant D1.

RESULTS

D1 gene polymorphisms and haplotypes in healthy Danish Twins

Characteristics of the study population are shown in Table 1, together with the genotype data of the two polymorphisms in the D1 gene. The minor allele frequencies are similar to those observed in other Caucasian populations (2, 4, 19).

The D1-C785T polymorphism was in a dose-dependent manner associated with higher serum rT3 as well as higher serum FT4 (Table 2). In addition, carriers of the D1-785T allele had a lower T3/T4 ratio and T3/rT3 ratio and a higher rT3/T4 ratio. The D1-A1814G polymorphism showed an opposite trend towards higher serum T3, a higher T3/rT3 and a higher T3/T4 ratio, however the associations failed to reach significance (Table 2). Power calculations indicated that population was large enough to observe an effect of this polymorphism on serum thyroid hormone levels. For instance, we could have detected a difference in the T3/rT3 ratio of at least 0.17 between non-carriers and carriers of the D1-A1814G polymorphism with α at 0.05 and β at 0.80.

Table 1: Characteristics of the population of Danish twins

		Danish Twins
N		1192
Age (yrs)		36.67 ± 10.72
Gender (M/F)		601/591
TSH (mU/L)		1.56 [1.14-2.17]
FT4 (pmol/L)		12.8 [11.6-13.8]
T4 (nmol/L)		111 [100 - 127]
T3 (nmol/L)		1.84 [1.66 - 2.09]
rT3 (nmol/L)		0.35 [0.29 - 0.43]
T3/rT3 (molar ratio)		5.32 [4.36 - 6.62]
T3/T4 (molar ratio)		1.65 [1.49 – 1.81]
rT3/T4 (molar ratio)		0.31 [0.26 - 0.36]
D1-C785T	wild-type (C/C) (%)	532 (44.6)
	heterozygote (C/T) (%)	516 (43.3)
	homozygote (T/T) (%)	144 (12.1)
	T-allele frequency (%)	33.7
D1-A1814G	wild-type (A/A) (%)	839 (78.0)
	heterozygote (A/G) (%)	231 (21.5)
	homozygote (G/G((%)	6 (0.5)
	G-allele frequency (%)	11.3

Data are shown as mean ± SD or as median and [interquartile range].

Table 2: Serum thyroid hormone levels by D1 genotypes in a population of Danish twins

	D1-C785T			P[a]	D1-A1814G		P[a]
	wild-type (532)	heterozygote (516)	homozygote (144)		wild-type (839)	carrier (231+6)	
TSH	1.72 ± 0.89	1.82 ±0.98	1.71 ±0.89	0.41	1.72 ± 0.93	1.84 ± 0.95	0.07
T4	114.9 ± 25.3	117.4 ± 25.7	117.1 ± 23.8	0.16	116.5 ± 25.0	117.3 ± 27.1	0.75
FT4	12.67 ± 1.61	12.89 ± 1.62	13.15 ± 1.71	0.004	12.85 ± 1.65	12.72 ± 1.55	0.30
T3	1.92 ± 0.39	1.91 ± 0.41	1.87 ± 0.32	0.12	1.90 ± 0.37	1.96 ± 0.45	0.07
FT3	6.21 ± 0.86	6.18 ± 0.89	6.15 ± 0.94	0.16	6.19 ± 0.91	6.22 ± 0.83	0.55
rT3	0.35 ± 0.10	0.37 ± 0.11	0.40 ± 0.12	<0.001	0.37 ± 0.11	0.36 ± 0.11	0.33
T3/rT3	5.88 ± 1.79	5.51 ± 1.78	5.09 ± 1.72	<0.001	5.53 ± 1.73	5.85 ± 1.89	0.05
T3/T4	1.70 ± 0.27	1.65 ± 0.28	1.63 ± 0.29	<0.001	1.66 ± 0.26	1.70 ± 0.31	0.07
rT3/T4	0.31 ± 0.08	0.32 ± 0.08	0.34 ± 0.09	<0.001	0.32 ± 0.08	0.31 ± 0.08	0.20

Data are shown as mean ± SD.

[a] P-value for linear regression on the transformed values, adjusted for age and gender.

The D1-C785T and D1-A1814G polymorphisms were analyzed for linkage disequilibrium and haplotypes were constructed. We observed three possible haplotype alleles in this population in the following frequencies: haplotype 1 (C-A) 55.6%, haplotype 2 (T-A) 33.1%, haplotype 3 (C-G) 11.3%. Haplotype 4 (T-G) was absent in 2152 chromosomes (Fig. 1). These frequencies were similar to those observed in other Caucasian populations (2, 19). Haplotype 1 (C-A) showed no association with serum thyroid hormone levels (data not shown). Hap-

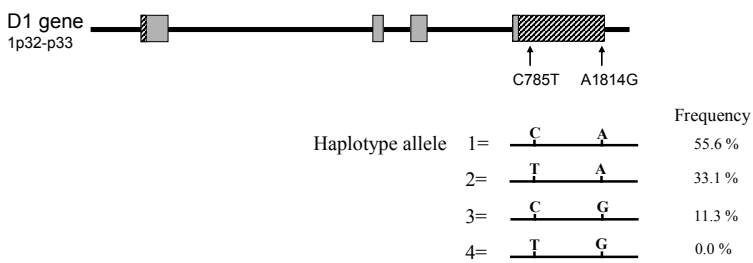

Figure 1: Location of the two polymorphisms in the *DIO1* gene (Chromosome 1p32-33, NM 000792) and the four haplotype alleles that were constructed from these polymorphisms, together with their frequencies in the Danish twin population. The coding region is represented by solid grey boxes, whereas dashed boxes indicate the untranslated regions.

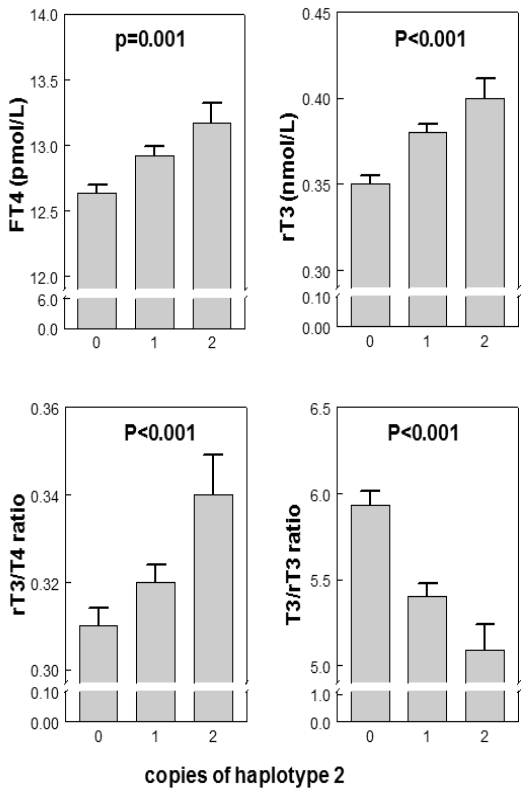

Figure 2: Differences in FT4 and rT3 levels and rT3/T4 and T3/rT3 ratios by haplotype 2. P-values represents linear regression on the transformed values, adjusted for age and gender. Data are shown as mean ± SEM.

Table 3: Heritability estimates with and without adjustment for the D1-C785T polymorphism

Phenotype	Number of individuals	Variation explained (%)	Heritability Estimate[a]	Heritability estimate[a] adjusted for D1-C785T polymorphism	P-value[b]
lnTSH	1192		0.6796 (0.61 - 0.73)	0.6795 (0.61 - 0.73)	0.36
ln Free T4	1192	0.87%	0.5979 (0.52 - 0.66)	0.5923 (0.58 - 0.59)	0.005
lnFree T3	1192		0.5845 (0.50 - 0.65)	0.5846 (0.50 - 0.65)	0.10
ln T4	1190		0.6087 (0.54 - 0.67)	0.6087 (0.54 - 0.67)	0.21
lnT3	1190		0.6044 (0.53 - 0.67)	0.6029 (0.53 - 0.67)	0.24
ln rT3	1190	1.79%	0.6658 (0.61 - 0.67)	0.6592 (0.60 - 0.71)	<0.001
lnT3rT3 ratio	1190	2.50%	0.7108 (0.66 - 0.76)	0.7027 (0.64 - 0.75)	<0.001
lnT3T4 ratio	1190	1.24%	0.6154 (0.61 - 0.62)	0.6092 (0.60 - 0.61)	<0.001
ln rT3/T4 ratio	1190	1.48%	0.7011 (0.64 - 0.73)	0.6956 (0.64 - 0.74)	0.001

[a] The proportion of the total variance attributable to total additive genetic variance. All heritability estimates are adjusted for age and gender

[b] Tested using a likelihood ratio chi-square test with 1 degree of freedom. The difference between minus twice the log-likelihood for a reduced model (in which the effect of the genotype is set to zero) and that of the full model (including genotype information) is approximately chi-square distributed.

lotype 2 (T-A) was associated with higher serum rT3, FT4 and rT3/T4 ratio and lower T3/rT3 ratio (Fig. 2). Only 6 subjects had 2 copies of haplotype 3 (C-G). Therefore, subjects carrying 1 and 2 copies were combined into carriers, whereas subjects with 0 copies were defined as non-carriers. Carriers had lower FT4 levels (p=0.07) and higher T3/rT3 ratio (p=0.05), however these associations failed to reach significance.

Quantitative genetic modeling

The use of this twin population allowed us to assess the contribution of these polymorphisms to the variation in serum thyroid variables. The D1-C785T polymorphism explained 0.87% and 1.79% of the variation in serum FT4 and rT3 respectively.

Since D1 plays an important role in the production of T3 from T4 and in the clearance of rT3, we expect polymorphisms in D1 to affect not only serum thyroid hormone levels, but in particular ratios between serum iodothyronines, such as the T3/rT3 ratio. The D1-C785T explained 1.48% and 2.50% of the variation in rT3/T4 and T3/rT3 ratios (Table 3). In addition, 1.24% of the variation in the T3/T4 ratio is accounted for by the D1-C785T polymorphism.

Since the associations between D1-A1814G and serum thyroid variables failed to reach significance, we refrained from calculating heritability estimates for this polymorphism.

mRNA stability of D1 variants

To analyze the underlying functional mechanism, we performed mRNA degradation rate measurements after blocking transcription with actinomycin D, as these polymorphisms in the 3'UTR might alter D1 mRNA stability. Transfected JEG3 cells were treated with actinomy-

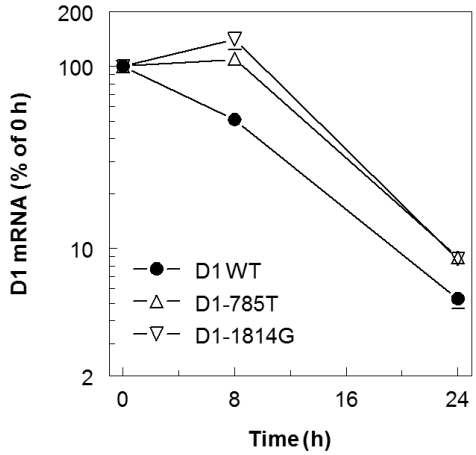

Figure 3: Decay rate of D1 mRNA levels after 0, 8 and 24 h of actinomycin D treatment. mRNA levels at 0 h are set at 100%. (100% corresponds to Ct-values ± SD of 18.97 ± 0.64 for D1-WT, 18.65 ± 0.56 for D1-785T and 19.84 ± 0.52 for D1-1814G respectively). Results are means ± SEM of three independent experiments. There was no significant difference in decay rate of wild-type D1, D1-785T or D1-1814G.

cin D for 0, 8 or 24 h. There were no significant differences in D1 mRNA levels between cells transfected with wild-type D1, D1-785T or D1-1814G cDNA (data not shown). In addition, there was little or no difference in D1 mRNA half-life between wild-type D1 and either one of the variants (Fig. 3).

rT3 metabolism by D1 variants

In addition to the mRNA stability experiments, we attempted to mimic *in vivo* D1 metabolism. Since the active site of D1 is located intracellulary (1), iodothyronines need to be transported across the plasma membrane for subsequent metabolism by D1. Therefore, JEG3 cells were co-transfected with MCT8 (14), an active thyroid hormone transporter which is also expressed in the liver, together with cDNA encoding for wild-type or variant D1. Subsequently, cells were incubated with [^{125}I]rT3 and analyzed for metabolism.

Cells transfected with MCT8 alone did not show significant metabolism of rT3 (data not shown). Co-transfection of cells with D1 and MCT8 greatly increased rT3 metabolism. However, no differences in rT3 metabolism were observed between wild-type D1, D1-785T or D1-1814G (Fig. 4).

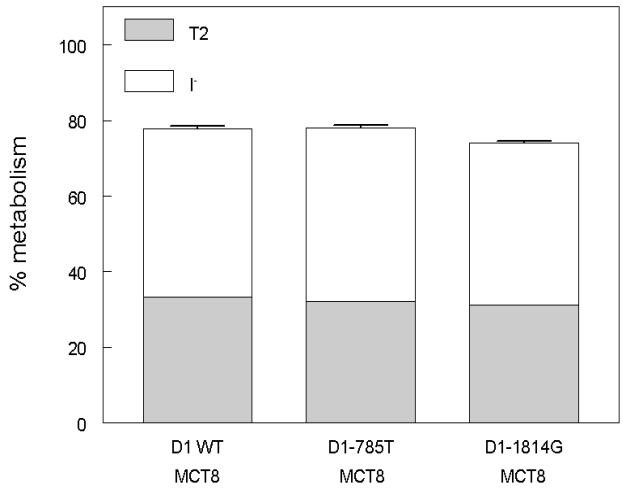

Figure 4: [^{125}I]rT3 metabolism by JEG3 cells transfected with wild-type D1, D1-785T or D1-1814G, together with MCT8. Cells were incubated for 4 h at 37 C with 1 nM [^{125}I]rT3. Metabolism is shown as percentage of metabolites in the medium. 100% metabolism corresponds to complete conversion of 1 nM [^{125}I]rT3 into [^{125}I]T2 and/or [^{125}I]I^-. Results are the means ± SEM of three experiments. There were no significant differences in metabolism between wild-type D1, D1-785T and D1-1814G.

DISCUSSION

Our study show that genetic variation in the D1 gene alters serum thyroid hormone levels in a cohort of healthy Danish twins. The D1-C785T polymorphism in particular was consistently and significantly associated with serum FT4, rT3, T3/rT3, T3/T4 and rT3/T4 ratios. Moreover, we have quantitated the proportion of genetic variation in serum thyroid variables accounted for by this polymorphism and analyzed the underlying functional mechanism.

D1 plays an important role in the conversion of T4 to T3. However, rT3 is the preferred substrate, reflecting the important role of D1 in the degradation of thyroid hormone (1). Therefore, one would expect polymorphisms in D1 to be associated with rT3 levels and to some extent also with T3 and T4 levels. In healthy Danish twins, carriers of the D1-C785T indeed had 3.8% higher FT4 levels than non-carriers, however T4 levels were not significantly different between wild-type and carriers of this polymorphism, probably reflecting differences in TBG in this population of both men and women. Carriers of the D1-C785T polymorphism had 14.3 % higher rT3. In addition, the D1-785T allele was associated with a lower T3/rT3 and T3/T4 ratio, whereas the rT3/T4 ratio was higher in carriers of the T allele. These findings are not only consistent with the physiological role of D1, but also with previous findings regarding this polymorphism and clearly minimize the risk of a false positive finding (2, 4, 19).

Although the D1-C785T polymorphism was consistently and significantly associated with serum thyroid hormone levels, our results also demonstrate that the proportion of genetic influence explained by this particular polymorphism is small. For instance, the D1-C785T polymorphism explained only 0.87% of the variation in serum FT4 levels. Thus, a substantial proportion of genetic variation, not accounted for by this polymorphism, still exists. This clearly suggests that multiple loci are involved in the control of serum thyroid variables. This is in agreement with the general notion that single polymorphisms usually have a subtle effect, while clinically significant effects generally result from many genes each contributing only slightly to the variance. Moreover, such a multi-gene organization may be regarded as an advantage for the overall control of thyroid homeostasis, which is tightly regulated and under this scenario not as vulnerable to genetic mutations when a few polymorphisms had a strong genetic impact.

Differences in ratios of serum iodothyronines might better reflect the effect of polymorphisms in D1 than differences in serum thyroid variables themselves (3). Serum thyroid hormone levels depend not only on the activities of the different deiodinases, but also on thyroid function and serum thyroid hormone-binding capacity. Because of the confounding effect of variable concentrations of thyroid hormone-binding proteins, the ratios of serum iodothyronines most accurately reflect the activity of the different deiodinases (20). For instance, a low D1 activity will result in reduced T3 production and rT3 clearance, and will thereby give rise to a lower T3/rT3 ratio. It is, therefore, interesting to note that the D1-C785T polymorphism explained 2.50% of the variation in T3/rT3 ratio, while the variation explained in T3/T4 ratio was relatively lower with 1.24%. This indicates that D1 expression has a larger impact on the serum T3/rT3 ratio than on the T3/T4 ratio, which is understandable in view of the quantitatively more important role of D1 in rT3 than in T4 metabolism.

The D1-A1814G polymorphism was not, or only borderline, significantly, associated with serum thyroid variables. Since this polymorphism is partially linked to D1-C785T, we performed haplotype-based analyses to determine which variant drives the associations. Only haplotype 2, i.e., D1-785T/1814A, was significantly associated with serum thyroid hormone levels, which demonstrates that D1-C785T is driving the associations and, undoubtedly, has a greater effect on serum thyroid hormone concentrations than the D1-A1814G polymorphism. This has been determined in different populations, including those recently reported by Panicker et al (2, 4, 21).

Since the D1-C785T and D1-A1814G polymorphism are located in the 3'UTR of the D1 mRNA, we hypothesized that these polymorphisms might influence D1 mRNA levels. For instance, Fang *et al.* have demonstrated that polymorphisms located in the 3'UTR of the vitamin D gene result in lower mRNA levels and increased mRNA decay rate (9). Therefore, we studied D1 mRNA stability in D1-transfected JEG3 cells, which were treated for 0, 8 or 24 h with actinomycin D. Although actinomycin D effectively reduced D1 mRNA levels after 24 hrs, we did not observe differences in mRNA level or decay rate between wild-type D1 or D1

carrying the C785T or A1814G polymorphisms. In addition, no differences were observed between wild-type or variant D1 at the protein level. Reverse T3 metabolism induced by wild-type or variant D1, in cells co-transfected with the thyroid hormone transporter MCT8 (14) was virtually identical. Since we did not observe any differences between wild-type and variant D1, this might suggest that these polymorphisms are linked to another functional polymorphism elsewhere in the *DIO1* gene. However, no polymorphisms in Hapmap (www.hapmap.org) in the coding region of the *DIO1* gene are in high linkage disequilibrium with our polymorphisms. Our polymorphisms could also be linked to variants located in the promoter region or in intronic regions of the *DIO1* gene. The latter may affect the splicing of the D1 transcript, and a variety of D1 splice variants have been documented (www.ncbi.nlm.nih.gov). Alternatively, the risk alleles of these 3'UTR polymorphisms might alter the binding sites of microRNA's, and thereby inhibit translation initiation or promote degradation of D1 mRNA (22, 23). However, both the C785T and the A1814G polymorphism did not interfere with predicted microRNA target sites in the 3'UTR of the *DIO1* gene (www.microRNA.org). Therefore, the mechanism underlying the association between the 3'UTR polymorphisms and D1 function remains to be established.

In conclusion, the D1-C785T polymorphism is consistently and significantly associated with serum thyroid hormone levels. However, the proportion of genetic influence explained by this particular polymorphism is small. No effect of the polymorphism on D1 mRNA levels, D1 mRNA decay rate or D1 activity was observed. The underlying functional mechanism needs to be elucidated, but might be explained by linkage to another polymorphism elsewhere in the *DIO1* gene.

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Chapter 9

Impact of thyroid function and polymorphisms in the type 2 deiodinase on blood pressure: the Rotterdam Study and the Rotterdam Scan Study

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ABSTRACT

Introduction: Thyroid function and genetic variation in the hypothalamic-pituitary-thyroid axis have been implicated in blood pressure regulation and susceptibility to hypertension. However studies conducted thus far were small with controversial results.

Objective: To examine whether serum thyroid parameters and polymorphisms in the type 2 deiodinase and the TSH receptor are associated with blood pressure and the presence of hypertension in two large cohorts of elderly subjects.

Design and participants: We studied a random sample of 1444 subjects of the Rotterdam study, and 997 subjects of the Rotterdam Scan study, two population-based cohort studies among elderly individuals aged 55-90 years.

Outcome measurements: Data on blood pressure and hypertension were obtained, and serum thyroid parameters, D2-Thr92Ala, D2-ORFa-Gly3Asp and TSHR-Asp727Glu polymorphisms were determined.

Results: In contrast to previous findings, no consistent and/or significant associations were found between serum TSH and FT4 and blood pressure in both cohorts. In addition, the D2-Thr92Ala, D2-ORFa-Gly3Asp and TSHR-Asp727Glu polymorphisms were not associated with blood pressure or the risk of hypertension.

Conclusions: In two large populations of elderly subjects, neither serum thyroid parameters nor polymorphisms in the type 2 deiodinase and the TSH receptor, were associated with blood pressure or the presence of hypertension. Our data suggest that thyroid function is not an important determinant of hypertension in elderly Dutch subjects.

INTRODUCTION

Adequate levels of thyroid hormone are essential for the regulation of many metabolic processes throughout life. Clinical manifestations of thyroid hormone excess, such as atrial fibrillation, illustrate the important role of thyroid hormone on the heart and vascular system (1). On the cellular level, triiodothyronine (T3) affects the heart via binding to nuclear T3 receptors in cardiomyocytes, thereby altering the transcription rate of T3-responsive genes, such as myosin heavy chains α and β. Additionally, T3 decreases vascular resistance via dilatation of arterioles of the peripheral circulation (1). This leads to a high cardiac output with enhanced systolic and diastolic function and low systemic vascular resistance in patients with hyperthyroidism. Hypothyroidism, on the contrary, is associated with decreased cardiac output, impaired systolic and diastolic function and increased systemic vascular resistance (1). In addition, vascular smooth muscle cells and endothelium play an important role in modulating vascular tone. Both are potential targets of thyroid hormone action and may be altered in the elderly (1).

Over the last years, several epidemiological studies have associated thyroid function with the occurrence of hypertension (2-4). In most cases, a positive association was found between thyroid stimulating hormone (TSH) and blood pressure in euthyroid subjects (2-4), although the relationship between subclinical thyroid disease and blood pressure is less clear-cut (5-8). More recently, genetic variation in the hypothalamic-pituitary-thyroid axis has been linked to susceptibility to hypertension (9, 10). The Thr92Ala polymorphism in the type 2 deiodinase (D2), which locally converts the pro-hormone T4 to the biological active T3, has been linked to blood pressure (10). This was, however, not confirmed in another study (11). Both studies were carried out in relatively small cohorts (n = 372, and n = 315 respectively) (10, 11).

We analyzed in two large cohorts of elderly subjects (n=1444 in the Rotterdam Study and n=997 in the Rotterdam Scan Study) whether serum TSH and free T4 (FT4) are associated with blood pressure and hypertension. Since previous genetic studies were carried out in relatively small cohorts, we also studied whether genetic variation in D2 and the TSH receptor (TSHR) is a determinant of hypertension susceptibility in these large populations.

SUBJECTS AND METHODS

Study populations

The Rotterdam Study is a population-based, prospective cohort study on chronic and disabling diseases in the elderly. A total of 7983 men and women, aged 55 years and over living in a district of Rotterdam, agreed to participate (12). In a random sample of 1544 subjects, serum levels of TSH and FT4 were determined. Of these, 1444 were included in the present study. Exclusion criteria were the use of thyroid medication (n=34) or missing information on

blood pressure, body mass index (BMI) or the use of anti-hypertensive medication (n=17). In addition, 49 subjects were excluded based on serum FT4 levels indicating clinical hypo- or hyperthyroidism (FT4 < 11.0 or > 25.0 pmol/L).

The Rotterdam Scan Study is an ongoing prospective population-based cohort study designed to investigate causes and consequences of age-related brain changes on MRI (13). In 1995 and 1996, subjects were randomly selected, in strata of sex and 10-years age groups, from the population-based Zoetermeer and Rotterdam studies (14, 15). Out of the 1077 individuals who participated, 997 were eligible for analysis in this study. Subjects were excluded when they were on thyroid medication (n=23) or had serum levels indicating hypo- or hyperthyroidism (n=46). Furthermore, 11 subjects had missing data on blood pressure, the use of anti-hypertensive medication or BMI.

The Rotterdam Study and the Rotterdam Scan Study were approved by the Medical Ethics Committee of Erasmus University Medical School and written informed consent was obtained from all participants.

Anthropometric and blood pressure measurements

For both cohorts, anthropometric measurements were obtained at the research center. BMI was calculated as weight in kilograms divided by height in meters squared. Blood pressure was measured twice with a random-zero sphygmomanometer with appropriate adult cuff size and the participant in sitting position. The average of 2 measurements was used for analyses. Pulse pressure was calculated as systolic blood pressure minus diastolic blood pressure. Information on the use of blood pressure-lowering medication and thyroid medication was obtained during a home interview. In both cohorts, hypertension was defined as a systolic blood pressure of 160 mm Hg or higher, a diastolic blood pressure of 100 mm Hg or higher or the use of blood pressure-lowering medication (16).

Thyroid hormone measurements

In the Rotterdam Study, TSH levels were measured with TSH Lumitest (Henning, Berlin, Germany). In the Rotterdam Scan Study, serum TSH was measured by a chemoluminescence assay (Vitros, ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK). Serum FT4 and T3 was measured by a chemoluminescence assay (Vitros). T3 was only determined in the Rotterdam Scan Study.

Genotyping

DNA was isolated from peripheral leukocytes according to standard procedures. Participants were genotyped for the D2-Thr92Ala (rs225014), the D2-ORFa-Gly3Asp (rs12885300) and the TSHR-Asp727Glu (rs1991517) polymorphisms. Genotypes were determined using the Taqman allelic discrimination assay (Assays-by-Design, www.appliedbiosystems.com). PCR cycling reactions were performed in 384 wells format in a 2 µl reaction volume on an ABI

9700 PCR system (Applied Biosystems Inc., Foster City, CA, USA). Results were analyzed by the ABI Taqman Prism 7900HT using the sequence detection system 2.22 software (Applied Biosystems). Five percent of samples were re-genotyped for assessment of genotyping accuracy. No inconsistencies were observed.

In the Rotterdam Study, DNA was available for genotyping in 1341 out of 1444 subjects. The genotyping success rate was 98.7% for D2-Thr92Ala, 99.1% for D2-ORFa-Gly3Asp and 98.0% for TSHR-Asp727Glu. In the Rotterdam Scan Study, DNA was available in 974 out of 997 subjects. Genotyping for D2-Thr92Ala failed in 4 subjects and for TSHR-Asp727Glu in 1 subject.

Statistical analysis

Data were analyzed using SPSS 10.0.7 for Windows (SPSS, Inc., Chicago, IL). P values are two-sided throughout and were considered significant if $P < 0.05$. For the polymorphisms, deviation from Hardy-Weinberg equilibrium proportions was analyzed using a Chi-square test. Linkage disequilibrium was assessed via the Haploview program (17). Haplotypes were constructed by the PHASE program (18).

Baseline characteristics between normotensive and hypertensive subjects were compared by χ^2 test or by analysis of variance, adjusted for age, gender and BMI if necessary. Age, gender and BMI adjusted odds ratios with their 95% confidence intervals (CI), were calculated using logistic regression analysis with hypertension as the dependent variable and serum TSH, FT4 or one of the polymorphisms as the independent variable. For both cohorts, serum TSH and FT4 were categorized into quintiles. The highest quintile of serum TSH and the lowest quintile of serum FT4 were used as reference category.

All subjects on blood pressure-lowering therapy were excluded for the analyses of the association of serum thyroid parameters and polymorphisms with blood pressure (n=432 in the Rotterdam Study and n=337 in the Rotterdam Scan Study). Serum TSH and FT4 were categorized into quintiles and linear regression analyses, adjusted for age, gender and BMI, were performed. For the polymorphisms, genotype-related differences were adjusted for age, gender and BMI and tested by analysis of covariance (ANCOVA).

RESULTS

The characteristics of both populations are shown in Table 1. Participants of the Rotterdam Study were on average 4 years younger than the participants of the Rotterdam Scan Study. In the Rotterdam Study almost 60 % was female, while in the Rotterdam Scan Study there was a one-to-one sex ratio. The allele frequencies of the polymorphisms were similar between the two populations and similar to those reported in other Caucasian populations (19, 20).

Table 1: Baseline characteristics of the Rotterdam Study and the Rotterdam Scan Study

Characteristic		Rotterdam Study	Rotterdam Scan Study
N		1444	997
Age (years)		68.84 ± 7.57	72.04 ± 7.33
Gender (female, %)		876 (60.7)	505 (50.7)
BMI (kg/m^2)		26.36 ± 3.57	26.67 ± 3.57
SBP (mm Hg)		138 ± 21	147 ± 22
DBP (mm Hg)		73 ± 11	79 ± 12
FT4 (pmol/L)		16.31 ± 2.66	17.75 ± 2.75
T3 (nmol/L)		-	2.02 ± 0.42
TSH (mU/L)		1.51 [0.99-2.30]	1.16 [0.73-1.76]
D2-Thr92Ala	wild-type	521 (40.1)	376 (38.8)
	heterozygote	634 (47.4)	459 (47.3)
	homozygote	169 (12.5)	135 (13.9)
	Ala-allele frequency	36.7%	37.6%
	HWE p-value	0.27	0.79
D2-ORFa-Gly3Asp	wild-type	533 (39.4)	414 (42.5)
	heterozygote	630 (47.9)	437 (44.9)
	homozygote	166 (12.8)	123 (12.6)
	Asp-allele frequency	36.2%	35.1%
	HWE p-value	0.34	0.65
TSHR-Asp727Glu	wild-type	1145 (87.1)	820 (84.3)
	heterozygote	166 (12.6)	149 (15.3)
	homozygote	3 (0.2)	4 (0.4)
	Glu-allele frequency	6.5%	8.1%
	HWE p-value	0.24	0.31

Data are shown as mean ± SD, or median [IQR] or as n, (%).

Serum thyroid parameters and blood pressure

Table 2 shows the differences in characteristics between hypertensive and normotensive subjects. Subjects who are using antihypertensive drugs are included in the group of hypertensive subjects. In both populations, subjects with hypertension were older and had a higher BMI than normotensive subjects.

Since serum TSH was positively associated with BMI and serum FT4 was inversely associated with BMI in our and other cohorts (21-23), and obese subjects were more prevalent in the hypertensive group, we adjusted for age, gender and BMI in the analysis of thyroid parameters and hypertension. In the Rotterdam Study, subjects with hypertension had significantly higher serum FT4 levels than normotensive subjects (Table 2). In addition, the prevalence of hypertension was increased in subjects with the highest FT4 levels (Table 3).

Several antihypertensive drugs (i.e. β-blockers and diuretics) are known to influence serum thyroid parameters (24, 25). For instance, diuretics are capable of binding to thyroid hormone-binding proteins and may thus interfere in FT4 assays (25). Therefore, we also performed an analysis after exclusion of all subjects on anti-hypertensive treatment (432 subjects from the Rotterdam Study and 337 participants from the Rotterdam Scan Study were excluded). Then, the prevalence of hypertension was not increased in subjects with the highest FT4

Table 2: Comparison between subjects with and without hypertension in the Rotterdam Study and the Rotterdam Scan Study

	Rotterdam Study		
	Hypertensive (575)	Normotensive (869)	P
Age (years)	70.75 ± 7.39	67.58 ± 7.44	<0.001
Gender (female, %)	363 (63.1)	513 (59.0)	0.12
BMI (kg/m^2)	27.30 ± 3.57	25.74 ± 3.43	<0.001
SBP (mmHg)	150 ± 23	131 ± 16	<0.001
DBP (mmHg)	77 ± 11	71 ± 10	<0.001
FT4 (pmol/L)	16.59 ± 2.77	16.13 ± 2.57	0.001[b]
TSH (mU/L)[a]	1.62 [1.00-2.46]	1.46 [0.98-2.21]	0.19[b]
Use of anti-hypertensive medication (N)	432	0	<0.001
	Rotterdam Scan Study		
	Hypertensive (502)	Normotensive (495)	P
Age (years)	73.66 ± 7.35	70.40 ± 6.95	<0.001
Gender (female, %)	265 (52.8)	240 (48.5)	0.17
BMI (kg/m^2)	27.37 ± 3.63	26.10 ± 3.35	<0.001
SBP (mmHg)	159 ± 22	136 ± 14	<0.001
DBP (mmH)	82 ± 13	75 ± 9	<0.001
FT4 (pmol/L)	17.88 ± 2.78	17.62 ± 2.71	0.25[b]
T3 (nmol/L)	2.01 ± 0.28	2.03 ± 0.53	0.62[b]
TSH (mU/L)[a]	1.24 [0.73-1.78]	1.11 [0.74-1.74]	0.85[b]
Use of anti-hypertensive medication (N)	337	0	<0.001

Data are shown as mean ± SD, or median [IQR] or as n, (%).

[a] Due to non-normal distribution, TSH is transformed by the natural logarithm.

[b] Data are corrected for age, gender and BMI.

levels (data not shown). In addition, no relation was observed between serum FT4 and blood pressure (Fig. 1A). Serum FT4 levels were, however, higher in participants on diuretic therapy compared to subjects with other anti-hypertensive treatment (subjects on diuretics vs. subjects on other anti-hypertensive treatment: 17.04 ± 0.21 vs. 16.37 ± 0.18 pmol/L, P=0.015).

In the Rotterdam Scan Study, similar associations were found between serum FT4 and blood pressure, however these failed to reach significance (Table 2, 3, Fig. 1B). No associations were found between serum TSH and blood pressure in both cohorts (Table 2, 3, Fig. 1). In addition, no associations were found between serum TSH and FT4 and pulse pressure, which is recognized as an independent risk factor for cardiovascular disease (data not shown) (26).

Triiodothyronine (T3), the active thyroid hormone, may have a more prominent role in the cardiovascular system than FT4 or TSH. However, serum T3 was not different between normotensive and hypertensive subjects of the Rotterdam Scan Study. In addition, no associations were found between serum T3 and the risk of hypertension (data not shown).

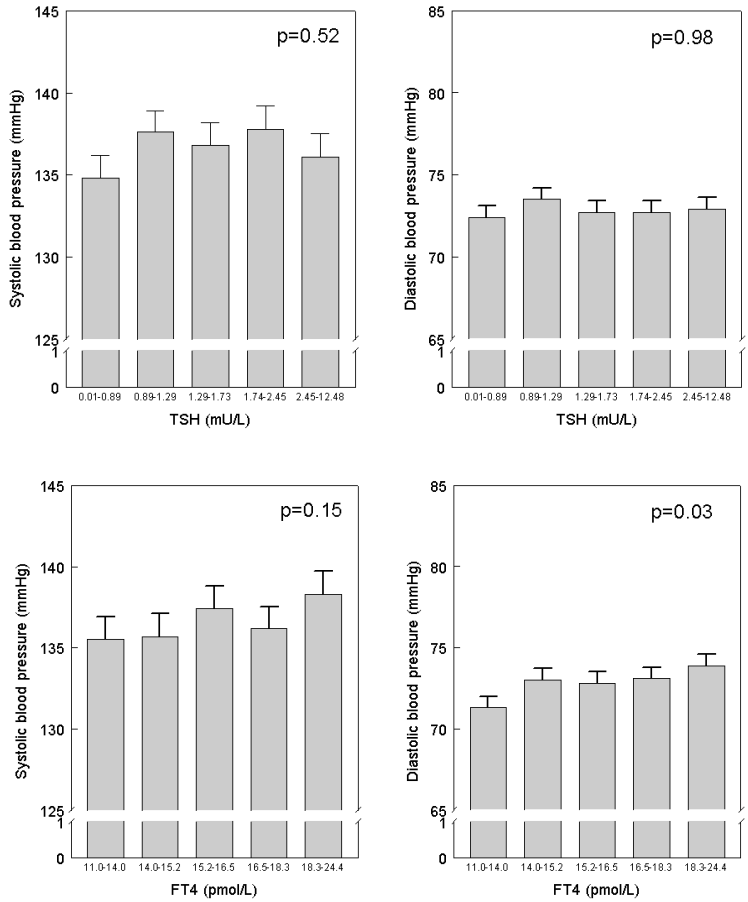

Figure 1A: Mean systolic and diastolic blood pressure (with SE) by categories of TSH and FT4, adjusted for age, gender and BMI in the Rotterdam Study

D2-Thr92Ala, D2-ORFa-Gly3Asp, TSHR-Asp727Glu, serum thyroid parameters and blood pressure

In both cohorts, serum TSH and FT4 were not different between wild-type, heterozygous or homozygous subjects for the D2-Thr92Ala and D2-ORFa-Gly3Asp polymorphisms, in line with previous findings in populations of elderly Caucasians (10, 20, 27) (data not shown). No differences in serum FT4 were observed between wild-type and carriers of the TSHR-Asp727Glu polymorphism. However, carriers of the TSHR-Glu^{727} allele had lower serum TSH levels than non-carriers (wild-type vs. carriers: 1.42 ± 0.04 vs. 1.36 ± 0.09 mU/L, P=0.06) (23).

Although the presence of hypertension was increased in an allele-dose dependent manner for the D2-ORFa-Gly3Asp polymorphism in the Rotterdam Scan Study, this same effect was not observed in the Rotterdam Study. In addition, risk of hypertension did not differ

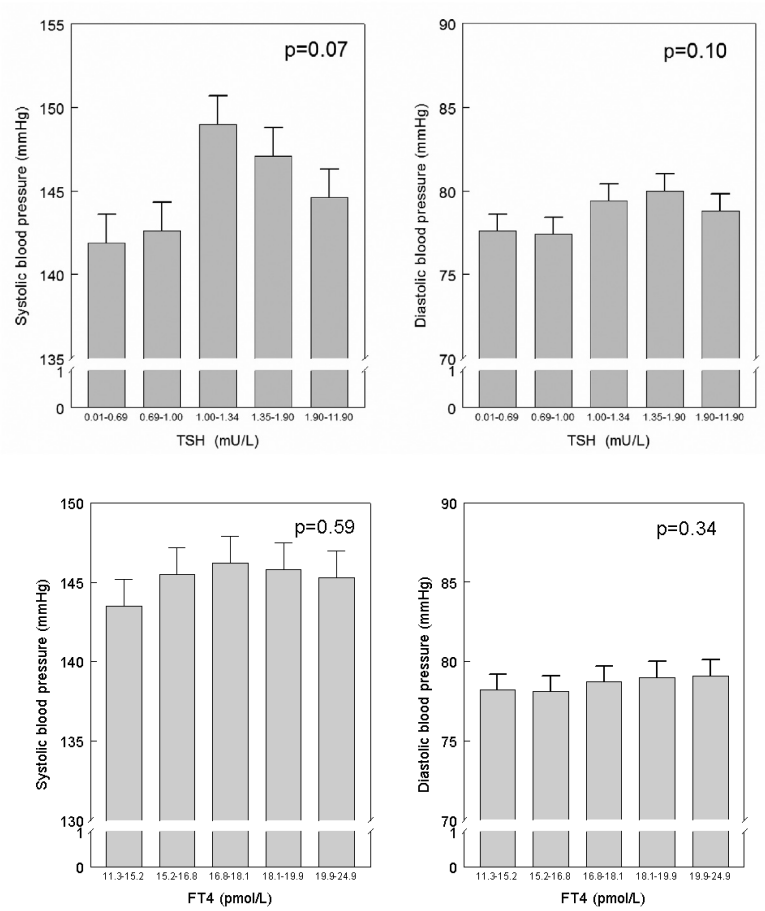

Figure 1B: Mean systolic and diastolic blood pressure (with SE) by categories of TSH and FT4, adjusted for age, gender and BMI in the Rotterdam Scan Study

by D2-Thr92Ala and TSHR-Asp727Glu genotype (Table 4). After exclusion of all subjects on blood pressure-lowering treatment, no associations between the polymorphisms and blood pressure were found (data not shown).

We analyzed the D2-Thr92Ala and D2-ORFa-Gly3Asp polymorphisms for linkage disequilibrium. In both cohorts, the D' was ~0.8 and frequency estimates of the 4 different haplotype alleles were similar to those reported in other Caucasian populations (20) (Fig. 2A and 2B). For the haplotypes, results of the association analyses were similar to those of the D2-Thr92Ala and D2-ORFa-Gly3Asp polymorphisms separately as the variant alleles are most often assigned to separate haplotypes (e.g. the Asp^{3} allele to haplotype 2 and the Ala^{92} allele to haplotype 3).

Table 3: Odds ratio of hypertension by TSH and FT4 quintiles in the Rotterdam Study and the Rotterdam Scan Study

Rotterdam Study		Rotterdam Scan Study	
TSH quintiles (mU/L)	**OR (95% CI)**	**TSH quintiles (mU/L)**	**OR (95% CI)**
1 (0.01-0.90)	0.75 (0.53-1.07)	1 (0.01-0.65)	1.10 (0.73-1.66)
2 (0.90-1.30)	0.82 (0.58-1.16)	2 (0.65-0.98)	0.72 (0.47-1.09)
3 (1.30-1.78)	0.73 (0.51-1.03)	3 (0.98-1.37)	0.89 (0.59-1.35)
4 (1.78-2.56)	0.85 (0.60-1.21)	4 (1.37-1.96)	0.96 (0.63-1.45)
5 (2.56-12.48)	Reference	5 (1.96-11.90)	Reference
	$P_{tr} = 0.13$		$P_{tr} = 0.83$
FT4 quintiles (pmol/L)	**OR (95% CI)**	**FT4 quintiles (pmol/L)**	**OR (95% CI)**
1 (11.0-14.0)	Reference	1 (11.0-15.3)	Reference
2 (14.0-15.3)	0.78 (0.54-1.11)	2 (15.3-16.9)	1.01 (0.67-1.52)
3 (15.3-16.7)	0.94 (0.66-1.34)	3 (16.9-18.4)	1.18 (0.78-1.78)
4 (16.7-18.5)	1.19 (0.84-1.68)	4 (18.4-20.2)	1.17 (0.78-1.77)
5 (18.5-25.0)	1.52 (1.07-2.15)	5 (20.2-25.0)	1.15 (0.76-1.74)
	$P_{tr} = 0.002$		$P_{tr} = 0.36$
No T3 data available		**T3 quintiles (nmol/L)**	**OR (95% CI)**
		1 (1.35-1.81)	Reference
		2 (1.81-1.94)	0.77 (0.51-1.16)
		3 (1.94-2.05)	1.12 (0.74-1.70)
		4 (2.05-2.18)	0.95 (0.62-1.44)
		5 (2.18-12.68)	1.02 (0.67-1.55)
			$P_{tr} = 0.60$

Data were adjusted for age, gender and BMI.

DISCUSSION

In this study we found no consistent associations between serum thyroid parameters and genetic variation in D2 and TSHR and blood pressure in two large populations of elderly Caucasians.

The cardiovascular system is a target organ for thyroid hormone action, which is apparent by the marked changes in cardiac function in patients with hypo-or hyperthyroidism. Serum TSH is positively associated with blood pressure in several population-based cohort studies (3, 4), although the associations observed were modest, with a rise of about 6 mmHg in systolic blood pressure within the reference range of serum TSH (3, 4) and the populations studied were large: Asvold and colleagues studied almost 35,000 subjects, while Iqbal *et al.* analyzed approximately 6,000 subjects of the Trømso study (3). The relationship between subclinical thyroid disease and blood pressure is less straightforward. Several reports have found significant associations between subclinical hypo- and hyperthyroidism and blood

Table 4: Odds ratio of hypertension by D2-Thr92Ala and D2-ORFa-Gly3Asp genotype

	Rotterdam Study		Rotterdam Scan Study	
	Cases/subjects	**OR (95% CI)**	**Cases/subjects**	**OR (95% CI)**
D2-Thr92Ala				
Wild-type	211/521	Reference	193/376	Reference
Heterozygote	245/634	0.94 (0.73-1.20)	247/459	1.11 (0.83-1.48)
Homozygote	69/169	1.09 (0.75-1.57)	55/135	0.66 (0.44-1.01)
		$P_{trend} = 0.90$		$P_{trend} = 0.21$
D2-ORFa-Gly3Asp				
Wild-type	218/533	Reference	194/414	Reference
Heterozygote	239/630	0.79 (0.62-1.01)	233/437	1.29 (0.97-1.71)
Homozygote	70/166	0.94 (0.65-1.36)	68/123	1.44 (0.96-2.26)
		$P_{trend} = 0.31$		$P_{trend} = 0.04$
TSHR-Asp727Glu				
Wild-type	452/1145	Reference	417/820	Reference
Carrier	68/169	1.04 (0.74-1.47)	77/153	1.13 (1.09-1.18)
		P = 0.83		P = 0.87

Data were adjusted for age, gender and BMI.

pressure (n=3233) (28, 29), whereas others have not been able to confirm this (n=2000, n=883, n=1510) (6-8).

In the Rotterdam Study, serum FT4 was significantly higher in hypertensive than normotensive subjects. Similarly, subjects in the highest FT4 quintile had a two-fold increased prevalence of hypertension compared to those in the lowest quintile. However, this effect disappeared after exclusion of subjects on antihypertensive treatment. Moreover, we could not confirm our findings between serum FT4 and blood pressure in the Rotterdam Scan Study. In addition, no consistent associations were found between serum TSH or T3 and blood pressure. However, it should be noted that with regard to serum TSH there was a difference in median TSH between the two populations, due to the use of different assays, which makes it more difficult to interpret the data.

Several factors might explain the discrepancy between our findings and previously published reports. First of all, the participants of the Rotterdam Study and the Rotterdam Scan Study had an average age of 68 and 72 years respectively. Since it is well known that blood pressure rises with increasing age (30, 31), it could well be that age has a much more pronounced effect on blood pressure than serum thyroid parameters in our study. Secondly, serum thyroid hormone levels were associated with BMI. Since obese subjects had a higher risk of hypertension, we adjusted for BMI in our analysis of serum thyroid parameters. However, only in some of the studies, the results were adjusted for BMI (2-4). Finally, the association between serum thyroid parameters and blood pressure appears to be quite modest (4).

Therefore, we cannot rule out that our populations were too small to observe such a relationship. However, since we were not able to demonstrate an association between serum thyroid parameters and blood pressure in these two cohorts, we conclude that thyroid function does not seem to have a clinically relevant contribution to blood pressure in Dutch elderly. Moreover, if thyroid function would have an important influence on blood pressure, opposite associations between serum TSH and FT4 and blood pressure should be observed due to the tight regulation of TSH and FT4 in the hypothalamic-pituitary-thyroid axis. However, in our cohorts, the associations pointed in the same direction. Therefore, we believe that there is no thyroidal effect on blood pressure in these two populations.

Based on the expression of D2 in the media of the aorta, human artery smooth muscle cells and vascular smooth muscle cells (32, 33), Gumieniak and colleagues proposed that polymorphisms in D2 might be associated with hypertension. Indeed, they showed that the Ala allele of the D2-Thr92Ala polymorphism was overrepresented in hypertensive versus normotensive subjects in a population of 372 subjects (10). In a study among type 2 diabetes mellitus patients, Canani and colleagues failed to show an association between the D2-Thr92Ala polymorphism and hypertension in 315 participants (11). Based on these studies and on the expression pattern of these proteins, (10, 32, 33), we analyzed the relationship between polymorphisms in D2 and TSHR and blood pressure. It would have been of interest to study polymorphisms in D1 as well, since D1 is another important source of serum T3 in humans. However, D1 is not expressed in heart or aorta, nor has it previously been associated with blood pressure. For this reason, we focused on polymorphisms in D2 and TSHR.

In our study, no associations were found between the D2-Thr92Ala and D2-ORFa-Gly3Asp polymorphisms and blood pressure in two large populations of elderly Caucasians. In addition, in agreement with Gumieniak *et al.* we observed no significant associations between TSHR-Asp727Glu polymorphism and blood pressure. It should be noted that there are important differences between the populations we studied and the cohort of Gumieniak *et al*. On average, the subjects in our populations were 30 years older than the participants in the cohort of Gumieniak *et al*. About 40-50% of the subjects of the Rotterdam Study and the Rotterdam Scan Study were hypertensive based on the World Health Organization (WHO) definition of hypertension (16). In the Gumieniak *et al.* report 82% of the participants were hypertensive based on criteria that were quite different from the WHO hypertension criteria we used. Unfortunately, we could not use the same definition for hypertension as Gumieniak *et al.* as no data on family members with hypertension were available in our cohorts. In addition, we did not stop anti-hypertensive treatment 2 to 4 weeks before the start of the study. This might explain these conflicting findings. However, we could have detected a relative risk for hypertension for the D2-Thr92Ala polymorphism of at least 1.5 in the Rotterdam Study, whereas in the Gumieniak *et al.* report the odds ratio for hypertension in carriers of the D2-Ala92 allele was 2.11 compared with non-carriers (10). Therefore, the inconsistency

between our data and the findings of Gumieniak *et al.* could be due to differences in population characteristics, phenotype definition or chance.

In conclusion, the results of our study indicate that neither serum TSH and FT4, nor the D2-Thr92Ala, D2-ORFa-Gly3Asp and TSHR-Asp727Glu polymorphisms are associated with blood pressure in euthyroid elderly Caucasians. Given the fact that there were no consistent and/or significant associations, our data do not support an important role for thyroid function and polymorphisms in thyroid hormone pathway genes in influencing blood pressure in elderly Dutch subjects.

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Chapter 10

A pathway analysis of genes involved in thyroid hormone synthesis, metabolism and transport using genome-wide association data of the Rotterdam Study

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Manuscript in preparation

ABSTRACT

Introduction: In healthy subjects, variability in circulating concentrations of thyroid hormone between individuals is greater than variability in the same individual sampled repeatedly over time. It is estimated that approximately 65% of this variation is due to genetic factors. The causative genes are, however, not well established.

Objective: To identify polymorphisms in known thyroid hormone pathway genes which influence serum TSH and FT4.

Design: We performed a pathway analysis of 67 genes involved in thyroid hormone synthesis, metabolism and transport using genome-wide association data of 1121 men and women of the Rotterdam Study. All subjects were euthyroid with negative thyroid peroxidase antibodies. Association analysis was done using linear regression in PLINK, adjusted for age and gender.

Results: For serum TSH, 2 polymorphisms located within the *THRB* gene showed significant association. In addition, 1 polymorphism in the *LRP2* gene and 1 in the *SCLO1B1* gene were also significantly associated with serum TSH. Four polymorphisms were significantly associated with serum FT4. Two were located within the *DIO1* gene and 2 were located within the *SLC7A8* gene.

Conclusions: This study has identified polymorphisms in the *THRB*, *LRP2* and *SLCO1B1* genes, which are associated with serum TSH levels. In addition, polymorphisms in the *DIO1* and *SLC7A8* genes are associated with serum FT4 levels. These findings provide insights into the molecular basis of population-based variation in serum TSH and FT4.

INTRODUCTION

Adequate levels of thyroid hormone are essential for normal development and growth, since thyroid hormone plays an important role in virtually all metabolic processes in the human body (1). This is clearly demonstrated in patients with thyroid hormone disorders. Hyperthyroidism, e.g., high serum levels of thyroid hormone, leads to symptoms such as palpitations, excessive sweating and weight loss (2-4). In contrast, decreased serum thyroid hormone levels due to hypothyroidism can result in weight gain, depression, atherosclerosis and hypertension (2, 5, 6).

In healthy subjects, variability in circulating concentrations of thyroid hormone between individuals is greater than variation in the same individual sampled repeatedly over time. In addition to environmental factors such as dietary iodine intake, genetic variation plays an important role in the control of the hypothalamic-pituitary-thyroid axis. It is estimated that approximately 65% of this variation is due to genetic factors (7-9). The causative genes are, however, not well established. Polymorphisms in the deiodinases (10-12), TSH receptor (12, 13), thyroid hormone transporters (14-16) have been associated with serum thyroid hormone levels, but their contribution to the overall variance is modest (17, 18). Recently, Panicker and colleagues performed a genome-wide linkage scan with 737 microsatellite markers in a large twin cohort (19). They identified eight chromosomal loci involved in the control of the pituitary-thyroid axis, but as expected from underpowered linkage scan in related subjects, they did not identify the actual genes explaining the variation in serum thyroid hormone levels. Arnaud-Lopez and colleagues recently performed a genome-wide association (GWA) study and demonstrated that polymorphisms in the Phosphodiesterase 8B gene are associated with serum TSH levels and thyroid function (20).

In this study we performed a focused analysis of thyroid hormone pathway genes in the context of a GWA study in elderly Caucasian men and women of the Rotterdam Study. We selected 67 genes based on their reported function in thyroid hormone synthesis, metabolism and transport and associated them with serum TSH and FT4 levels. Four polymorphisms, of which 2 in the *DIO1* gene and 2 in the *SLC7A8* gene, were associated with serum FT4 ($P<0.001$). For serum TSH, analyses revealed several polymorphisms in the *THRB, LRP2* and *SLCO1B1* genes with significant association. These data provide convincing evidence for an important role of these genes in thyroid hormone action and justify further clinical and biological investigation.

MATERIALS AND METHODS

Study population

The cohort consisted of a sample of 1121 men and women from the Rotterdam Study, an ongoing prospective population-based cohort study, focused on chronic disabling conditions of the elderly in the Netherlands. A total of 7983 men and women, aged 55 years and over living in a district of Rotterdam, agreed to participate (21). In a random sample of 1544 subjects, serum levels of TSH and FT4 were determined. Of these, 1121 were included in the present study. Exclusion criteria were missing information on genome-wide genotype data and/or the use of thyroid medication (n=228). In addition, 195 subjects were excluded based on TPO-antibodies > 10 IU/L and/or serum FT4 levels indicating clinical hypo- or hyperthyroidism (FT4 < 11.0 or > 25.0 pmol/L).

Anthropometric and thyroid hormone measurements

Anthropometric measurements were obtained at the research center. BMI was calculated as weight in kilograms divided by height in meters squared. Serum TSH levels were measured with TSH Lumitest (Henning, Berlin, Germany). Serum FT4 was measured by a chemoluminescence assay (Vitros). TPO-antibodies were assessed by ELISA (Milenia, DPC, Los Angeles, USA) and regarded as positive when greater than 10 IU/L.

Selection of candidate genes

Sixty-seven genes were selected on the basis of their reported function in thyroid hormone, synthesis and metabolism. This selection was made by searching NCBI Genbank for 'thyro' limited by 'human' and 'current'. Via this method 520 genes were identified. Based on the current literature, a specialist in the field (Theo Visser) reviewed these genes and came to a final selection of 69 genes that were proven to be involved in thyroid hormone synthesis, metabolism and transport. Since our cohort consisted of both men and women, genes on the X-chromosome (i.e., MCT8 and TBG) were removed prior to analysis, resulting in a total of 67 genes (Table 1).

Statistical methods

The Rotterdam Study was genotyped using the Illumina HumanHap 550K array, and quality control (QC) was performed using PLINK (22). After QC 561,466 polymorphisms (with a minimal sample call-rate of 97.5%) in 1121 men and women were available for analysis. After selecting polymorphisms with a minor allele frequency >=5%, HWE $p > 1 \times x10^{-6}$, a genotyping call-rate of at least 90%, and within a 10 Kb region of one of the 67 candidate genes (n=1297 polymorphisms), association analysis was done using linear regression in PLINK (22), adjusted for age and gender. Due to non-normal distribution, TSH was transformed by the natural logarithm. In GWA studies, analysing over 500,000 polymorphisms, a stringent

Table 1: Selection of candidate genes involved in thyroid hormone synthesis, metabolism and transport

Gene code	Name	ID	Chr	Start	Stop	Start - 10kb	Stop + 10kb
ALB	Albumin	213	4	74488870	74505996	74478870	74515996
CD36	Fatty acid translocase	948	7	80069459	80144262	80059459	80154262
CGA	Glycoprotein hormone alpha subunit	1081	6	87851941	87861543	87841941	87871543
CRYM	Mu-crystallin	1428	16	21177343	21221918	21167343	21231918
DIO1	Type I deiodinase	1733	1	54132449	54149347	54122449	54159347
DIO2	Type II deiodinase	1734	14	79733622	79748278	79723622	79758278
DIO3	Type III deiodinase	1735	14	101097441	101099542	101087441	101109542
DIO3OS	Opposite strand of type III deiodinase	64150	14	101088313	101091766	101078313	101101766
DUOX2	Dual oxidase 2	50506	15	43172144	43193651	43162144	43203651
DUOXA2	Dual oxidase maturation factor 2	405753	15	43193816	43197596	43183816	43207596
FOXE1	Forkhead box E1	2304	9	99655358	99658818	99645358	99668818
GPHA2	Glycoprotein hormone alpha 2	170589	11	64458519	64459936	64448519	64469936
GPHB5	Glycoprotein hormone beta 5	122876	14	62849395	62854316	62839395	62864316
IYD	DEHAL1	389434	6	150731721	150767457	150721721	150777457
LRP2	Megalin	4036	2	169691865	169927368	169681865	169937368
NKX2-1	Thyroid transcription factor 1	7080	14	36055353	36059167	36045353	36069167
NKX2-5	NK2 transcription factor related, locus 5	1482	4	42436076	42438776	42426076	42448776
NR1D1	Rev-Erb alpha	9572	17	35502567	35510499	35492567	35520499
PAX8	Paired box 8	7849	2	113690045	113752968	113680045	113762968
RXRA	Retinoid X receptor alpha	6256	9	136358231	136472252	136348231	136482252
RXRB	Retinoid X receptor beta	6257	6	33269343	33276410	33259343	33286410
RXRG	Retinoid X receptor gamma	6258	1	163636974	163681054	163626974	163691054
SECISBP2	SECIS binding protein	79048	9	91123232	91164381	91113232	91174381
SLC10A1	NTCP	6554	14	69312305	69333707	69302305	69343707
SLC16A10	MCT10	117247	6	111515502	111650907	111505502	111660907
SLC26A4	Pendrin	5172	7	107088316	107145490	107078316	107155490
SLC3A2	4F2hc	6520	11	62380094	62412929	62370094	62422929
SLC5A5	NIS	6528	19	17843782	17865897	17833782	17875897
SLC5A8	Apical iodide transporter	160728	12	100073402	100128120	100063402	100138120
SLC7A5	LAT1	282369	18	11470909	11499821	11460909	11509821
SLC7A8	LAT2	23428	14	22664344	22722689	22654344	22732689
SLCO1A2	OATP1A2	6579	12	21311651	21439638	21301651	21449638
SLCO1B1	OATP1B1	10599	12	21175404	21283997	21165404	21293997
SLCO1B3	OATP1B3	28234	12	20854905	20960925	20844905	20970925
SLCO1C1	OATP1C1	53919	12	20739666	20797587	20729666	20807587

table continued on next page

Gene code	Name	ID	Chr	Start	Stop	Start - 10kb	Stop + 10kb
SLCO2A1	OATP2A1	6578	3	135134230	135231418	135124230	135241418
SLCO3A1	OATP3A1	28232	15	90197950	90507783	90187950	90517783
SLCO4A1	OATP4A1	28231	20	60744242	60774092	60734242	60784092
SLCO4C1	OATP4C1	353189	5	101597589	101660152	101587589	101670152
SLCO5A1	OATP5A1	81796	8	70747129	70909762	70737129	70919762
SLCO6A1	OATP6A1	133482	5	101735550	101862619	101725550	101872619
SULT1A1	Sulfotransferase 1A1	6817	16	28524414	28542367	28514414	28552367
SULT1A2	Sulfotransferase 1A2	6799	16	28510767	28515892	28500767	28525892
SULT1A3	Sulfotransferase 1A3	6818	16	30113244	30123151	30103244	30133151
SULT1B1	Sulfotransferase 1B1	27284	4	70627275	70661019	70617275	70671019
SULT1C2	Sulfotransferase 1C2	6819	2	108271527	108292803	108261527	108302803
SULT1C4	Sulfotranferase 1C4	27233	2	108360853	108370702	108350853	108380702
SULT1E1	Sulfotransferase 1E1	6783	4	70741519	70760459	70731519	70770459
SULT4A1	Sulfotransferase 4A1	25830	22	42551720	42589711	42541720	42599711
TG	Thyroglobulin	7038	8	133948387	134216325	133938387	134226325
THRA	Thyroid hormone receptor alpha	7067	17	35472589	35503646	35462589	35513646
THRB	Thyroid hormone receptor beta	7068	3	24134709	24511317	24124709	24521317
THRSP	Thyroid hormone responsive protein	7069	11	77452555	77457045	77442555	77467045
TPO	Thyroid peroxidase	7173	2	1396242	1525502	1386242	1535502
TRH	Thyrotropin-releasing hormone	7200	3	131176253	131179470	131166253	131189470
TRHDE	Thyrotropin-releasing hormone degrading enzyme	29953	12	70952730	71345689	70942730	71355689
TRHR	Thyrotropin-releasing hormone receptor	7201	8	110168900	110200989	110158900	110210989
TSHB	Thyroid stimulating hormone beta	7252	3	27736279	27741514	27726279	27751514
TSHR	Thyroid stimulating hormone receptor	7253	14	80491622	80682399	80481622	80692399
TTF1	Thyroid transcription factor 1	7270	9	134240756	134272042	134230756	134282042
TTR	Transthyretin	7276	18	27425838	27432781	27415838	27442781
UGT1A1	Glucuronyltransferase 1A1	54658	2	234333658	234346684	234323658	234356684
UGT1A10	Glucuronyltransferase 1A10	54575	2	234333658	234346684	234323658	234356684
UGT1A3	Glucuronyltransferase 1A3	54659	2	234302512	234346684	234292512	234356684
UGT1A7	Glucuronyltransferase 1A7	54577	2	234255323	234346684	234245323	234356684
UGT1A8	Glucuronyltransferase 1A8	54576	2	234191030	234346684	234181030	234356684
UGT1A9	Glucuronyltransferase 1A9	54600	2	234245283	234346690	234235283	234356690

p-value of $<5 \times 10^{-7}$ is usually required to correct for multiple comparisons. In this study, we carried out a focused analysis of 67 genes (1297 SNPs), and therefore a P-value$< 1{,}0 \cdot 10^{-3}$ was found to be significant.

RESULTS

Characteristics of the study population

The mean age ± SD of the 1121 subjects was 68.92 ± 7.56 years. Out of the 1121 subjects, 466 (41.6%) was male. The mean FT4 concentration was 16.4 ± 2.7 pmol/liter, whereas the median TSH (with interquartile range) was 1.45 (0.97 –2.15) mU/liter.

Association of polymorphisms in candidate genes with serum TSH and FT4

The analysis was carried out in 1121 elderly Caucasian men and women of the Rotterdam Study. For serum TSH, analyses revealed 4 polymorphisms with P-values <0.001. Two out of these 4 were located within the *THRB* gene (Table 2). In addition, one polymorphism in the *LRP2* and one in the *SCLO1B1* gene were also significantly associated with serum TSH. Moreover, several other polymorphisms throughout the *THRB* gene showed strong association with serum TSH; however, these did not reach the set threshold for significance.

Four polymorphisms were significantly associated with serum FT4. Two were located within the *DIO1* gene and 2 were located within the *SLC7A8* gene. Interestingly, several other polymorphisms within the *SLC7A8* gene were also strongly associated with serum FT4 levels (Table 3).

Interestingly, four genes came up for both serum TSH and FT4. These were *THRB*, *LRP2*, *FOXE1* and *SLCO1B1*.

DISCUSSION

In this study we used a pathway analysis of genes involved in thyroid hormone synthesis, metabolism and transport, to study the effects of common genetic variation in these genes on serum TSH and/or FT4. Polymorphisms in the *THRB, LRP2 and SLCO1B1* genes were found to be associated with serum TSH levels with P-values $<1{,}0 \cdot 10^{-3}$. In addition, variants in the *DIO1* and *SLC7A8* genes were associated with serum FT4.

Studies performed over the recent years have established that many factors determine thyroid hormone action and can contribute to the inter-individual variation in serum thyroid hormone levels. In addition to environmental factors such as smoking and dietary iodine intake, genetic variation influences thyroid hormone action (7). For instance, the TSHR-Asp727Glu polymorphism has been linked to lower serum TSH levels in up to 5 different

Table 2: Top polymorphisms associated with serum lnTSH from the pathway analysis based on GWA data from the Rotterdam Study ($P<1,0•10^{-2}$)

Chr	Polymorphism	Position	Minor allele	Beta	P-value	Gene
2	rs12988804	169826057	A	-0.1387	$9,2•10^{-5}$	LRP2
2	rs10490130	169807357	C	-0.1668	$7,0•10^{-3}$	LRP2
3	rs6550858	24384219	G	-0.1408	$1,6•10^{-4}$	THRB
3	rs6792725	24495287	A	0.1146	$9,9•10^{-4}$	THRB
3	rs13097208	24235063	A	-0.1016	$2,2•10^{-3}$	THRB
3	rs13066296	24149163	A	-0.1355	$3,4•10^{-3}$	THRB
3	rs12639293	24202179	A	-0.09827	$4,3•10^{-3}$	THRB
3	rs17014487	24319631	C	-0.09215	$4,5•10^{-3}$	THRB
3	rs9862985	24193883	A	-0.09671	$5,4•10^{-3}$	THRB
3	rs7636335	24255952	C	0.09705	$6,4•10^{-3}$	THRB
3	rs7625035	135210556	G	0.1066	$8,9•10^{-3}$	THRB
3	rs4858582	24194525	A	-0.08545	$9,4•10^{-3}$	THRB
12	rs964615	21220691	A	0.1838	$4,0•10^{-4}$	SLCO1B1
12	rs16923154	20777198	G	0.1777	$2,9•10^{-3}$	SLCO1C1
12	rs974453	20781209	A	0.1639	$4,4•10^{-3}$	SLCO1C1
14	rs10131915	80550040	C	0.4501	$3,1•10^{-3}$	TSHR
1	rs16844019	163632777	A	-0.5656	$4,6•10^{-3}$	RXRG
9	rs1443434	99657300	C	0.08805	$5,8•10^{-3}$	FOXE1
12	rs4309196	71026529	C	0.1007	$6,7•10^{-3}$	TRHDE
12	rs11568563	21348701	C	0.1784	$8,9•10^{-3}$	SLCO1A2
20	rs6011628	60783951	G	-0.1087	$9,2•10^{-3}$	SLCO4A1

Table 3: Top polymorphisms associated with serum FT4 from the pathway analysis based on GWA data from the Rotterdam Study ($P <1,0•10^{-2}$)

Chr	Polymorphism	Position	Minor allele	Beta	P-value	Gene
14	rs10145863	22706879	A	0.7075	$9,3•10^{-5}$	SLC7A8
14	rs1884544	22727441	G	0.7781	$4,5•10^{-4}$	SLC7A8
14	rs2268878	22689742	C	0.4512	$2,1•10^{-3}$	SLC7A8
14	rs2331937	22683680	A	0.4057	$3,0•10^{-3}$	SLC7A8
14	rs927041	22682426	A	0.3932	$3,9•10^{-3}$	SLC7A8
1	rs2235544	54148158	C	-0.4203	$1,3•10^{-4}$	DIO1
1	rs11206244	54148289	A	0.4298	$1,9•10^{-4}$	DIO1
6	rs17606253	111633138	G	0.4747	$2,5•10^{-3}$	SLC16A10
6	rs9400467	111530708	G	-0.349	$5,3•10^{-3}$	SLC16A10
3	rs4563353	24465133	A	-0.6288	$2,6•10^{-3}$	THRB
3	rs9845983	24491068	A	-0.6835	$8,2•10^{-3}$	THRB
2	rs4667591	169711678	A	0.3978	$3,3•10^{-3}$	LRP2
2	rs7568568	169843461	G	0.521	$7,5•10^{-3}$	LRP2
2	rs10210408	169755096	G	0.3171	$8,4•10^{-3}$	LRP2
12	rs964615	21220691	A	-0.5045	$5,3•10^{-3}$	SLCO1B1
9	rs1443434	99657300	C	-0.2973	$8,0•10^{-3}$	FOXE1

cohorts of Caucasian subjects (12, 13, 17, 23, 24). Moreover, different studies have convincingly shown that polymorphisms in the *DIO1* gene are associated with serum FT4 and rT3 levels and alter the T3/rT3 ratio (11, 12, 18, 25). The D1-C785T (rs11206244) is associated with 3.8% higher serum FT4, 14.3% higher rT3 levels and a lower T3/rT3 ratio, pointing towards a lower D1 activity in carriers of the T allele (18). Recently, Panicker and colleagues showed that the C-allele of the rs2235544 polymorphism in the *DIO1* gene is associated with increased D1 function resulting in 8.3% lower FT4 levels and an 8.6% increased T3/T4 ratio with GWA-wide levels of significance in 3976 individuals (25). In this study we demonstrate that these two polymorphisms are significantly associated with serum FT4, but not serum TSH in 1121 elderly Caucasian men and women. Since these findings are consistent with not only the physiological role of D1, but also with the previous findings regarding these polymorphisms, this minimizes the risk of false positive findings. In addition, it convincingly demonstrates that D1 has an important role in thyroid hormone metabolism.

Two polymorphisms within the *SLC7A8* gene, encoding LAT2, were also significantly associated with serum FT4 levels. In addition, several other polymorphisms within the *SLC7A8* gene were also strongly associated with serum FT4 levels, but these did not reach the set threshold for significance. Injection of *Xenopus Laevis* oocytes with cRNA for the LAT1 transporter stimulated the Na^+-independent uptake of several iodothyronines with Km values of 7.9 μM for T4, 0.8 μM for T3, 12.5 μM for rT3, 7.9 μM for 3,3'-T2 (26). Smaller increments in iodothyronine uptake were noted after injection of LAT2 cRNA (26). Based on the findings in this study, it seems worthwhile to study the role of LAT2 in thyroid hormone transport in more detail. In addition, no studies have been conducted to link polymorphisms in the *SLC7A8* gene to alterations in thyroid hormone transport that could as a consequence lead to altered serum thyroid hormone levels. Furthermore, it might be interesting to search for mutations in the *SLC7A8* gene in patients who have elevated serum TSH and FT4 levels.

The role of genetic variation in the deiodinases (10, 27) the TSH receptor (12, 17) and thyroid hormone transporters (16) has been studied in depth over the recent years. Interestingly, this does not hold true for polymorphisms in the thyroid hormone receptors. Many studies have shown that polymorphisms in other nuclear receptors, such as the estrogen and glucocorticoid receptors, play a role in age-related disorders, such as dementia and osteoporosis (28-30). Thyroid hormone receptors are important in the negative feedback-loop of the hypothalamus-pituitary-thyroid axis. Therefore, polymorphisms in the *THRB* gene might be associated with serum thyroid parameters. Recently, we identified several polymorphisms in the *THRB* gene. To our surprise, only one of these polymorphisms, e.g., THRB-in9-G/A polymorphism, was associated with 8.9% higher serum TSH levels in 1116 healthy Danish twins (31). In this study, a number of polymorphisms within the *THRB* gene were associated with serum TSH levels. These findings suggest that genetic variation in thyroid hormone receptor β plays an important role in determining an individual's set point of pituitary-thyroid axis. However, these findings are preliminary and need to be replicated in different large cohorts.

Our study has several strengths and limitations. We performed our analysis in a large cohort of both Caucasian men and women, which is representative for the general population. We excluded all subjects with thyroid disease or auto-immunity. Therefore, our data are not biased by gender or underlying disease. In addition, we have been able to replicate previous associations between polymorphisms in the *DIO1* gene and serum FT4. However, we have not yet replicated these novel associations in an independent cohort cohort. Thus, the risk alleles we have identified in the *THRB* and *SLC7A8* genes justify further clinical and biological investigations.

In conclusion, we performed a focused analysis of thyroid hormone pathway genes in the context of a genome-wide association analysis in elderly Caucasian men and women of the Rotterdam Study. In this cohort, polymorphisms in the *DIO1* and *SLC7A8* gene were associated with serum FT4 levels. In addition, polymorphisms in the *THRB* gene were associated with serum TSH levels. These findings require replication, but they are beginning to provide insights into the molecular basis of population based variation These findings provide insights into the molecular basis of population-based variation in serum TSH and FT4.

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Chapter 11

General Discussion

Thyroid hormone is essential for many metabolic processes in the human body (1), which is clearly demonstrated in patients with hypo- or hyperthyroidism. Even small alterations in serum thyroid hormone levels result in an increased risk to develop osteoporosis and can cause changes in heart rate or mood (2-5). This thesis focuses on the effect of variation in thyroid hormone pathway genes on serum thyroid hormone levels and clinical endpoints. In addition, associations between serum thyroid parameters and clinical endpoints, such as osteoporosis and hypertension, were analyzed. In this chapter the main findings of the studies described in this thesis are brought together and placed in a broader perspective. Methodological considerations, strengths and limitations of the research described in this thesis are discussed. Finally, the relevance and potential clinical implications are considered and suggestions for future research are put forward.

THE ROLE OF THE OATP1 SUBFAMILY IN THYROID HORMONE TRANSPORT

Based on the lipophilic structure of thyroid hormone, it was long assumed that thyroid hormone enters the cell through simple passive diffusion (6). However, with the discovery of MCT8 as a specific and active thyroid hormone transporter and the fact that mutations in this transporter lead to the Allan-Herndon-Dudley syndrome (OMIM 300523), the interest in this area of research has greatly increased (7-9). In this thesis we focused on several members of the OATP1 subfamily as potential thyroid hormone transporters. All four members of the OATP1 subfamily, OATP1A2, OATP1B1, OATP1B3 and OATP1C1, are located on the short arm of chromosome 12 and form a gene cluster together with a pseudogene. They are highly homologous and show a similar genomic organization, i.e., in all four transporter genes the open reading frame is composed of 14 exons. As demonstrated in this thesis, these transporters are capable of thyroid hormone transport, and exhibit overlapping substrate specificities.

Based on the different affinities for iodothyronines and the specific expression patterns, distinct physiological roles for these proteins are worthwhile considering. OATP1C1 shows narrow substrate specificity: Pizzagalli and colleagues demonstrated that it only transports T4 and rT3 (10). We extended these findings by showing that T4S uptake is also facilitated by OATP1C1. Together with the almost exclusive expression at the blood-brain barrier, this suggests that OATP1C1 is critical for T4 uptake into the brain. This important role is substantiated by Sugiyama and colleagues who showed that expression levels of OATP1C1 in isolated rat brain capillaries are regulated by thyroid hormone concentrations (11). OATP1C1 is up-regulated in hypothyroid rats and down-regulated in hyperthyroid rats (11). Together with changes in D2 expression (12), OATP1C1 counteracts the effect of alterations in serum T4 to ensure stable thyroid hormone concentrations in the brain. Recently, OATP1C1 homologues have been identified in chicken and mice (13, 14). Similar to its human ortholog, mice OATP1C1 transports T4 and rT3 with high affinity and is widely expressed in brain, suggesting

an important role in T4 transport across the blood-brain barrier (14). Nakao and colleagues demonstrated that also in chicken OATP1C1 transports T4.

Whether findings regarding the role of OATP1C1 in mice, rats and chicken can be extrapolated to humans, remains up to debate. However, due to the high level of homology, expression pattern and substrate specificity between rodent and human OATP1C1, it seems plausible that OATP1C1 is also important for T4 uptake in the human brain. This is supported by our data showing that polymorphisms in the OATP1C1 gene are associated with fatigue and depression in a cohort of hypothyroid patients (15). However, apart from T4 transport across the blood-brain barrier, no specific function of OATP1C1 in humans has yet been identified. In addition, no patients with a mutation in the OATP1C1 gene have thus far been identified. Loss of OATP1C1 function could possibly lead to a thyroid hormone related neuronal disorder characterized by resistance to thyroid hormone treatment (11). It seems worthwhile to study this in OATP1C1 knockout mice.

In contrast to OATP1C1, OATP1B1 and OATP1B3 are exclusively expressed in liver and share approximately 80% amino acid sequence identity with each other (16, 17). Both transporters have broad substrate specificities as they transport numerous compounds such as bilirubin, bile salts, drugs such as pravastatin and digoxin, but also steroid hormones (18). Many studies have been published on the role of these 2 proteins in drug absorption, distribution, and excretion (19, 20). They are involved in the cellular uptake of drugs into the liver, which is, besides the intestine and the kidneys, important for pharmacokinetics. In this thesis, we have demonstrated that OATP1B1 strongly induces uptake of iodothyronine sulfates T4S, T3S and rT3S, but has only little T4, T3 and rT3 transport activity (21). Like OATP1B1, OATP1B3 preferentially transports the sulfated iodothyronines and rT3. Under normal conditions, D1 in liver rapidly degradates rT3 and sulfated iodothyronines, keeping the serum concentrations of these substrates low (22-27). However, serum iodothyronine sulfate and rT3 levels are high in preterm infants and during critical illness, possibly due to decreased degradation (23, 25, 28). It is therefore of interest that both OATP1B1 and OATP1B3 only facilitate transport of the iodothyronine sulfates and rT3. This reflects their role of transporters of endo- and xenobiotics that are metabolized in the liver and excreted in the bile.

Finally, OATP1A2 is unique in transporting all (sulfated) iodothyronines. OATP1A2 facilitates transport T4, T3 and rT3. In addition, T4S, T3S and rT3S uptake is induced in OATP1A2 transfected cells compared to mock transfected cells. OATP1A2 is expressed in multiple tissues, among which liver, brain and kidney (29, 30). Based on its expression pattern, OATP1A2 could play a role in delivery of thyroid hormone across the blood-brain barrier. Alternatively, it could play a role in the removal of thyroid hormone from different tissues for degradation and eventual elimination via bile or urine. However, the Km values of OATP1A2-mediated T4 and T3 transport are far above the physiological serum concentration. Therefore, it does not seem likely that there is an important role for OATP1A2 in thyroid hormone transport in a physiological situation. However, it may serve as a back-up transport system in case other

transporters malfunction or are saturized, or thyroid hormone concentrations are higher than under normal conditions. The function of OATP1A2 in thyroid hormone transport could be studied in a knock out mice model, although this might be complicated as OATP1A2 is capable to transport a wide range of substrates. Therefore, the phenotype of such a mouse might be the net result of impaired uptake of not only thyroid hormone, but also of other substrates, such as estrogen (30).

POLYMORPHISMS IN THYROID HORMONE PATHWAY GENES AND SERUM THYROID HORMONE LEVELS

Virtually all studies described in thesis examine the effect of genes involved in thyroid hormone metabolism and transport on serum thyroid hormone levels and clinical endpoints using a genetic approach. Therefore, it is worthwhile to make some more general comments regarding genetic association studies.

The unraveling of the sequence of the human genome has led to a big expansion of research conducted in this area. Especially, studies on polymorphisms have expanded rapidly. This has lead to the publication of many genetic association studies with positive findings. However, only few of them have been replicated in other cohorts, usually with a much smaller effect size than the one described in the original paper (31, 32). This suggests that many of these initial studies were false positive. There are different ways to reduce false positive findings. First of all, it starts with reliable genotyping and phenotyping. Especially, in large-scale population studies, problems can arise due to the large amount of data and the many researchers involved. In addition, if different definitions for the same phenotype are used, the chance of non-replication increases. Therefore, standardization and stringent criteria should be used, although certain diseases may show variable phenotypic expression. A second requirement is a strong a priori hypothesis. For instance, we tested whether the OATPs were capable of thyroid hormone transport, prior to analysis of potential associations between polymorphisms in these genes and serum thyroid hormone levels. Since all these proteins transported iodothyronines in a significant manner, associations between polymorphisms in the genes encoding these transporters seems plausible.

Another possibility to reduce false-positive findings is to validate findings from the initial 'training' cohort in a second 'validation' cohort. Preferentially, both cohorts are large, thereby minimizing any power issues that might arise while studying polymorphisms with low minor-allele frequencies. In addition, the populations should be homogenous with regard to age, gender, ethnicity and environment. For instance, differences in age might cause non-replication. We can expect that the effect of a polymorphism is present at all ages, since a variant exists throughout life. However, genes can also be expressed and even function differently at different ages, e.g., genes that regulate puberty and/or menopause. In addition,

false-positive findings can also occur due to admixture. Frequencies of polymorphisms can differ between different ethnic groups. Except for the cohort of Danish twins, all populations studied in this thesis are of Dutch origin. Finally, it is important to realize that we do not completely understand interactions between environment and genetics in complex traits. Therefore, it is important to minimize differences between populations with regard to environment.

Genetic association studies are always faced with the issue of multiple testing. Performing a large number of tests will increase the number of false positive results. When should we correct for multiple testing? And if the answer to the former question is yes, how should we do this? By a simple, but conservative Bonferroni correction or by another method? In the case of a strong a priori hypothesis and associations that all point in the same direction, the issue of multiple testing can probably be dealt with easily. However, often this is not the case. Another way to substantiate any observed association is to confirm the *in vivo* results *in vitro*. An example of a study in which many of the above mentioned requirements are met, is the study of the OATP1B1-Val174Ala polymorphism. This polymorphism has been studied extensively, as OATP1B1 facilitates uptake of many drugs from the bloodstream into the liver. Previously, Niemi and colleagues have shown that this polymorphism leads to decreased function of OATP1B1 and thereby increases the systemic bioavailability of lipid lowering drugs (33). We have demonstrated that OATP1B1 preferentially transports sulfated hormones, i.e., T4S, T3S, rT3S and E1S (21). Therefore, we expected that the OATP1B1-Val174Ala polymorphism would be associated with serum levels of iodothyronine sulfates and E1S. Indeed, this polymorphism was associated with higher serum T4S levels in 155 blood donors, while in a larger cohort of elderly Caucasians this same polymorphism was associated with 40% higher E1S levels. Moreover, OATP1B1-Val^{174} showed a 40% higher induction of transport and metabolism of these substrates than OATP1B1-Ala^{174} in transfected COS1 cells (21) (Table 1).

However, regarding polymorphisms in other OATPs, the results were less clear-cut. OATP1C1 is capable of T4, T4S and rT3 transport, but polymorphisms in the OATP1C1 gene were not consistently associated with serum thyroid hormone levels. In blood donors the OATP1C1-C3035T was associated with higher FT4 and rT3 levels, but we failed to replicate this in a larger population of Danish twins (34). This inconsistency might be explained by many things including differences between the two populations. Iodine intake and conditions for serum sampling varied between these two groups. It is known that small differences in iodine intake are associated with altered thyroid function (35). According to the World Health Organization, Denmark is moderately iodine deficient, whereas The Netherlands has an adequate iodine intake (31). However, associations between variants in other thyroid hormone pathway genes and serum thyroid parameters, initially found in Dutch cohorts, have been replicated in the cohort of Danish twins (36). Moreover, no effect of the OATP1C1-C3035T polymorphism *in vitro* was observed either (Table 1). With regards to OATP1A2 and OATP1B3, no significant associations were observed between polymorphisms in these genes and serum thyroid hor-

mone levels (Table 1). Whether this means that these transporters do not play a significant physiological role in thyroid hormone transport or that the selection of polymorphisms we studied was not adequate, remains up to debate. We preferentially chose non-synonymous polymorphisms and polymorphisms located in the 3'UTR. Although functional mechanisms are more easily comprehensible when studying this kind of polymorphisms, the risk of missing an effect is apparent as single polymorphisms usually have subtle effects. Therefore, a better strategy might be to capture the complete genetic variation of the gene of interest, using a tagging approach. However, this increases the number of genotypes and haplotypes, and thereby the number of tests performed. In addition, the definition of where a gene starts and stops is unclear, in particular for regulatory elements.

Due to disappointing results of studies that employ a candidate gene approach, more and more studies use a GWA strategy, which is made possible through rapid technical progress over the recent years. A GWA approach allows identification of genetic risk alleles without prior knowledge of function. In Chapter 10 we used this method to identify genetic variants associated with serum TSH and FT4. We performed a pathway analysis of 67 genes involved in thyroid hormone synthesis, metabolism and transport using GWA data of 1121 men and women of the Rotterdam Study and identified polymorphisms in the *THRB*, *LRP2* and *SLCO1B1* genes which were associated with serum TSH levels. In addition, polymorphisms in the *DIO1* and *SLC7A8* genes were associated with serum FT4 levels. Since loci associated with complex diseases are generally assumed to have a modest effect, GWA studies require large sample sizes and due to multiple testing require replication of the association signals in a different cohort. This strategy has proven to be successful in the identification of genetic variants associated with different diseases, such as age-related macular degeneration (37), BMD and osteoporosis (38), type II diabetes (39) and cancer (40). However, it is important to realize that GWA studies have similar problems as candidate-gene approach based studies, such as failure to replicate (32, 41, 42). In addition, even though these studies can pinpoint a region of interest, they cannot unambiguously identify the causal genes. Therefore, resequencing and fine-mapping of the area of interest, followed by functional studies is still required. However, it has been shown to be a very succesful approach to identify previously unknown biological pathways involved in complex genetic traits.

EFFECT SIZE OF POLYMORPHISMS ON SERUM THYROID HORMONE LEVELS

Genetic variation in the TSHR and D1 affects normal thyroid hormone metabolism, as has been shown in different Caucasian populations. For instance, the Asp727Glu polymorphism in the TSHR is associated with lower TSH levels (36, 43, 44), whereas the D1-C785T polymorphism is associated with higher rT3 levels and a lower T3/rT3 ratio. Yet, the D1-A1814G polymorphism is associated with a higher T3/rT3 ratio (43, 45). In addition, Panicker and colleagues recently

demonstrated that another polymorphism in D1, rs2235544, is significantly associated with serum thyroid hormone levels in different populations (46). However, the effect size of these variants on serum thyroid hormone levels is not clear. In a large population of healthy Danish twins, we quantified the contribution of these polymorphisms to the genetic variance in serum thyroid hormone levels. Although the TSHR-Asp727Glu polymorphism was associated with decreasing serum TSH levels, this variant accounted for only 0.91% of the total phenotypic variance in serum TSH (36). In this same population, carriers of the D1-785T allele had 3.8% higher FT4 and 14.3 % higher rT3 levels, resulting in a lower T3/T4 and T3/rT3 ratio and a higher rT3/T4 ratio (47). However, the proportion of genetic variation of serum rT3 and FT4 explained by the D1-C785T is small, in the order of 1 to 2%. Therefore, a substantial proportion of variation, accounted for by other common or rare genetic variants, still exists. In addition, part of this variation might be explained by gene-gene interactions or gene-environment interactions. It suggests that multiple loci are involved in the control of serum thyroid variables, which is in agreement with the general notion that single polymorphisms usually have subtle effects. Such a multi-gene control may be regarded as an advantage for the overall control of thyroid hormone homeostasis, which is tightly regulated and under this scenario not as vulnerable to single gene mutations compared to when only a few genes were involved.

POLYMORPHISMS IN THYROID HORMONE PATHWAY GENES AND CLINICAL ENDPOINTS

The question arises whether polymorphisms are relevant, when the effect size of individual variants on serum thyroid hormone levels is so small. Therefore, polymorphisms in thyroid hormone pathway genes have been studied for association with many different clinical endpoints (Table 2). For instance, the D1-C785T polymorphism has been related to increased muscle strength and muscle mass (48). Recently, this polymorphism has also been linked with enhanced potentiation of the antidepressant effect of sertraline by T3 as reflected by declining HRSD-21 scores over an 8-week period (Cooper-Kazaz et al., accepted for publication). Based on these findings, together with the consistent associations of the D1-C785T polymorphism with serum thyroid hormone levels, we feel that this genetic variant is clinically relevant, albeit with a modest effect. The mechanism underlying the association between the D1-C785T polymorphism and D1 function remains to be established. We tested the functionality of this polymorphism in transfected JEG3 cells, but no effect of the D1-C785T polymorphism on D1 mRNA decay rate or D1 activity was observed. This might mean that this polymorphism is linked to another functional polymorphism elsewhere in the *DIO1* gene. For instance, it is linked to rs2235544, a polymorphism in intron 3 of the *DIO1* gene, which has recently been associated with serum thyroid hormone levels in different populations (46). Alternatively, the

Table 1: Effect of common variation in OATP1A2, OATP1B1, OATP1B3 and OATP1C1

Gene	Polymorphism	Location	Change	Effect on serum thyroid hormone levels	*In vitro* effect
OATP1A2	rs57921276	Exon	Ile13Thr	No effect observed	No effect observed
	rs57550534	Exon	Glu172Asp	No effect observed	Decreased transport capacity
OATP1B1	rs4149056		Val174Ala	Higher bilirubin, T4S, E1S levels, lower T3/rT3 ratio	Decreased transport capacity
OATP1B3	rs4149117	Exon	Ser112Ala	No effect observed	Not determined
	rs7311358	Exon	Met233Ile	No effect observed	Not determined
OATP1C1	rs10770704	Intron	C/T	No effect observed	Not determined
	rs36010656	Exon	Pro143Thr	Higher rT3 levels, however effect not consistent	No effect observed
	rs10444412	3'-UTR	C3035T	Higher FT4 and rT3 levels, however effect not consistent	No effect observed

D1-C785T polymorphism might cause an alteration in the binding sites of microRNA's, and thereby inhibit translation initiation or promote degradation of D1 mRNA (49, 50).

Many studies have been conducted to link the polymorphisms in the *DIO2* gene to thyroid hormone related clinical endpoints. For instance, polymorphisms in the *DIO2* gene have been associated with mental retardation in iodine-deficient areas (51). Guo and colleagues showed that the prevalence of genotypes of the *DIO2* polymorphisms was different between children who were mentally retarded and children with a normal IQ in an iodine-deficient region of China. In addition, de Jong et al. published a study looking at the relationship between polymorphisms in the *DIO1* and *DIO2* genes and MRI features of Alzheimers disease. No associations were found between these polymorphisms and early MRI markers of Alzheimers dementia (45) (Table 2).

Several studies have addressed the effect of *DIO2* polymorphisms on response to thyroid hormone replacement. Appelhof et al. showed that there was no difference in preference for thyroxine monotherapy or combination therapy with T4 and T3 between carriers and non-carriers of the *DIO2* polymorphisms (52) (Table 2). Recently, Torlontano demonstrated that in thyroidectomized patients carriers of the D2-Thr92Ala polymorphism require higher thyroxine dosages to acquire the same TSH levels than non-carriers (53). Based on these conflicting data, new studies are needed to confirm or to reject these findings.

The D2-Thr92Ala polymorphism has also been associated with diabetes and hypertension in some studies (54-56), but these findings could not be replicated in our and in other studies (57, 58). In two large cohorts, we tested whether this polymorphism was associated with hypertension, but no association was observed (Table 2). In addition, no relationship between serum thyroid hormone levels and blood pressure was found either.

There is a clear relationship between thyroid function and bone. In addition to direct effects of FT4 on the skeleton, TSH has been shown to have a direct effect on bone as well (59,

60). TSHR knockout mice die by 10 weeks of age and display high-turnover osteoporosis, which supplementation with thyroid hormone fails to reverse. TSH inhibits through the TSHR both osteoclastic bone resorption and osteoblastic bone formation. In the Rotterdam Study, we first analyzed whether serum TSH and FT4 levels were associated with bone parameters. In line with the effect of TSH on bone in mice, serum TSH showed a positive trend with BMD in humans. This is strengthened by the association between the TSHR-Asp727Glu polymorphism and femoral neck BMD we observed in the same population (Table 2). However, serum FT4 had a much greater influence on bone than TSH (44). Recently, Heemstra *et al*. performed a cross-sectional study with 148 patients, who had been thyroidectomized for differentiated thyroid carcinoma to study the relationship between serum TSH and indicators of bone turnover (61). They found a significant, inverse correlation between serum TSH levels and indicators of bone turnover, which was independent of serum FT4 and T3 levels as well as other parameters influencing bone metabolism. In addition, carriers of the TSHR-Asp727Glu polymorphism had an 8.1% higher femoral neck BMD, which was, however, no longer significant after adjusting for BMI (61) (Table 2). Besides a relationship between thyroid hormone and bone, Meulenbelt and colleagues have identified *DIO2* as a susceptibility gene conferring risk to osteoarthritis (62). Osteoarthritis is a common late-onset articular joint disease for which no pharmaceutical intervention is available to attenuate the cartilage degeneration. Meulenbelt performed a genome-wide linkage scan and combined linkage association analysis. They demonstrated that female carriers of a common haplotype in the *DIO2* gene, exclusively containing the minor allele of rs225014 and the common allele of rs12885300 polymorphism, had an odds ratio of 1.79 for developing advanced/symptomatic hip osteoarthritis (62) (Table 2).

Besides endpoints known to be directly related to thyroid hormone metabolism, it is also worthwhile to consider whether polymorphisms in thyroid hormone pathway genes are associated with other clinical endpoints. For instance, polymorphisms in OATP1B3 are associated with impaired testosterone transport and improved survival in patients with prostate cancer (63). In addition, OATP1B3 polymorphisms have been studied in relationship with pharmacokinetics of paclitaxel, an anti-cancer agent (64, 65). Similar to OATP1B3, polymorphisms in OATP1A2, but especially in OATP1B1 are associated with pharmacokinetics of different drugs (19, 33, 66). Various studies have demonstrated that polymorphisms in the OATP1B1 gene can lead to altered transport characteristics or protein localization, which can contribute significantly to inter-individual variations of drug effects. For instance, the OATP1B1-Val174Ala polymorphism shows decreased transport activity and leads to a higher area under the curve for different drugs such as statins or anti-cancer agents (67, 68). Recently, this same polymorphism has been associated with an increased risk of statin-induced myopathy. Genotyping this variant may help to achieve the benefits of statin therapy more safely and effectively (69). This points to the notion that, although we are still far from using genetic variants in daily clinical practice, probably genetic variants will be first used in individualized drug therapy as more and more evidence is accumulating that polymorphisms contribute significantly to dif-

Table 2: Effect of common variation in thyroid hormone pathway genes on thyroid hormone related clinical endpoints

Gene	Effect on thyroid hormone related clinical endpoints	Effect consistent?
DIO1	IGF-1, muscle strength and lean body mass (48)	replication required
	Potentiation of the antidepressant effect of sertraline by T3	replication required
	Alzheimers disease (45)	replication required
DIO2	Osteoarthritis (61)	replication required
	Hypertension (54-58)	No
	Diabetes (54-58)	No
	Psychological well-being in patients on thyroxine	replication required
	Response to combination therapy in patients on thyroxine (52, 53)	No
	Mental retardation (51)	replication required
	Alzheimers disease (45)	replication required
OATP1C1	Psychological well-being in patients on thyroxine (15)	replication required
TSHR	Bone mineral density (44,61)	Yes
	Insulin resistance / metabolic parameters (57)	replication required

ferences in patients' drugs responses. For instance, Schwarz et al. showed that initial variability in the international normalized ratio (INR) response to warfarin was strongly associated with genetic variability in the pharmacologic target of warfarin, VKORC1 (70). In addition, genetic variants in thiopurine methyltransferase, uridine diphosphate glucuronosyltransferase and dihydropyrimidine dehydrogenase have been helpful in predicting the efficacy and toxicity of anti-cancer treatment (71). Polymorphisms in immune response genes are known to influence individuals' susceptibility to infectious diseases and the severity of those diseases. Likewise, genetic variation in bacteria or virusses can influence the severity of the disease or the likelihood of colonization. For instance, persistent carriage of *S. aureus* is influenced by genetic variation in host inflammatory response genes (72). The host genotype was also associatd with carriage of certain *S. aureus* genotypes. In addition, polymorphisms in the hepatitis C virus are used as predictive parameters to define the antiviral treatment strategy and the chance of therapeutic success (73).

FUTURE STUDIES

The role of OATPs in thyroid hormone transport has been analyzed in this thesis. It is worthwhile to discuss directions for further research regarding the OATPs. For instance, OATP1B1 and OATP1B3 are important for the hepatic uptake of different drugs. Therefore, we wonder what would happen with serum T4S, T3S or rT3 levels in patients on statin therapy. In addition, besides transport of sulfated iodothyronines, we and others have demonstrated that OATP1B1 transports E1S (17, 21, 74, 75). In this thesis, we show that carriers of the OATP1B1-Val174Ala polymorphism have almost 40% higher E1S levels than non-carriers (21). In contrast to the role of sulfation in thyroid hormone metabolism, the sulfation of estrogens is readily revers-

ible by estrogen sulfatase (76). The formation of E1S is thought to serve as a reservoir for active estrogen, since serum concentrations of E1S are 10-20 times higher than those of the unconjugated estrogens (77). Estrogens play an important role in breast cancer, i.e., estrogen is the main hormone involved in the development and growth of breast tumors. Therefore, therapies aim to block interaction with the estrogen receptor by use of an anti-estrogen, or by inhibiting the conversion of androstenedione to estrone with an aromatase inhibitor (78, 79). Local formation of estrogens in breast tumors might be more important than circulating estrogens for growth and survival of estrogen-dependent breast cancer in post-menopausal women (80). Serum E1S is a major source of estrogens for breast tissue through local sulfatase activity (81). The role of OATP1B1 in the risk of developing breast cancer should therefore be investigated, since carriers of the OATP1B1-Val174Ala polymorphism have life-long higher serum E1S levels (21).

Another direction for future research worthwhile investigating, concerns the expression levels of OATP1C1 during fetal development. Sugiyama et al. already demonstrated that OATP1C1 expression in rats is regulated by thyroid hormone (11). Based on these and other findings, we speculated that OATP1C1 is important for T4 uptake across the blood-brain barrier. If we would find significant expression levels of OATP1C1 in several brain regions from early fetal life onward, this would substantiate this hypothesis.

Several polymorphisms in thyroid hormone pathway genes have been associated with variation in serum thyroid parameters and clinical endpoints. This holds true for the deiodinases (48, 82) the TSH receptor (36, 43) and thyroid hormone transporters (83). The role of genetic variants in the thyroid hormone receptors (TRs) is unknown, while many studies have been published on associations between clinical endpoints and polymorphisms in other nuclear receptors, such as the estrogen and glucocorticoid receptors. TRs are important in the negative feedback-loop of the hypothalamus-pituitary-thyroid axis. Patients with rare and severe mutations in TRβ display a phenotype of thyroid hormone resistance with elevated serum FT4 and non-suppressed TSH levels. Therefore, common and more subtle polymorphisms in the *THRB* gene might be associated with serum thyroid parameters. Recently, we identified several polymorphisms in the *THRB* gene. To our surprise, only one of these polymorphisms was associated with serum TSH levels (84). However, a candidate gene analysis of thyroid hormone synthesis, metabolism and transport showed that many polymorphisms in the *THRB* gene were associated with serum TSH levels (Chapter 10). Therefore, the next step would be to study the *THRB* gene in relationship with thyroid hormone parameters and thyroid hormone-dependent endpoints.

Although many patients with mutations in TRβ have been described, no patients with mutations in TRα have been identified yet. However, various mouse strains with mutations in TRα are viable (85-87). Depending on the location of the mutation, these mice exhibit extreme anxiety-like behavior, osteosclerosis, bradycardia, or signs of altered metabolism (e.g. obesity, increased fasting insulin levels). Intriguingly, in mice part of these symptoms

can be relieved by treatment with thyroid hormone (85). Although likely, it is currently not known whether genetic variation in TRα has similar effects in humans. Therefore, it is of interest to study the consequences of genetic variation in the *THRA* gene, e.g., by searching for patients with mutations in the *THRA* gene and performing genetic association studies with *THRA* polymorphisms.

Besides the classical candidate gene approach, GWA analysis will be very useful to identify novel loci involved in the regulation of thyroid hormone levels. Although a GWA study is complicated, as it requires large samples sizes, replication and reliable geno- and phenotyping, it will unravel previously unknown pathways involved in thyroid hormone metabolism. Panicker et al. identified several loci associated with serum FT4 and TSH by a genome-wide linkage scan with 737 microsatellite markers (88), but as expected from an underpowered linkage scan in related subjects, they did not identify the actual genes explaining the variation in serum thyroid hormone levels. Arnaud-Lopez and colleagues recently demonstrated that polymorphisms in the Phosphodiesterase 8B gene are associated with serum TSH levels and thyroid function (89). Probably, GWA studies will provide more candidate genes involved in thyroid function.

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Chapter 12

Summary - Samenvatting

SUMMARY

The thyroid gland, situated in front of the thyroid cartilage in the neck, consists of two lobes and only weighs a few grams in adults. The thyroid produces mainly T4 (thyroxine), an inactive form of thyroid hormone. T4 can be converted into T3, the active thyroid hormone, by enzymes called deiodinases, like the type 1 and 2 deiodinase (D1 and D2). The biological activity of thyroid hormone is amongst others dependent on the presence of thyroid hormone in the blood stream, the binding of T3 to its nuclear T3 receptor, the activity of intracellular deiodinases, and the presence of thyroid hormone transporters.

Thyroid hormone is essential for normal development and growth of virtually all tissues. Too much thyroid hormone, e.g., hyperthyroidism, leads to weight loss, restlessness, heat-intolerance and osteoporosis. Hypothyroidism, decreased thyroid hormone levels, can lead to fatigue, weight gain, cold-intolerance or even depression. Even subtle changes in serum thyroid hormone levels can have important consequences on thyroid hormone related endpoints, such as atherosclerosis, heart rate, depression and osteoporosis. The studies described in this thesis show that small variations in genes involved in thyroid hormone synthesis, transport and metabolism influence serum thyroid hormone levels and thyroid hormone dependent endpoints, such as normal bone development. The background of thyroid hormone production, metabolism and transport are introduced in **Chapter 1**. In addition, the aims of this thesis are presented in this chapter.

In **Chapter 2** the role of OATP1B1 as a thyroid hormone transporter was analyzed. In addition, the role of the OATP1B1-Val174Ala polymorphism was studied. Although OATP1B1 did not facilitate uptake of T4 and T3, it is an important factor in transport and metabolism of bilirubin, E1S and iodothyronine sulfates. Moreover, carriers of the OATP1B1-Val174Ala polymorphism have higher bilirubin, E1S and T4S levels than non-carriers, since the OATP1B1-Ala^{174} displays 40% decreased transport activity. **Chapter 3** reports on the function of OATP1C1, another member of the OATP1 subfamily, which is expressed almost exclusively at the blood-brain barrier. OATP1C1 transports T4, rT3 and to some extent also T4S. Taken together with a study from Sugiyama and co-workers, in which they show that OATP1c1 is up-regulated in hypothyroid rats and down-regulated in hyperthyroid rats, we feel OATP1C1 plays a significant role in thyroid hormone transport across the blood-brain barrier. Genetic variants in this transporter gene are not associated with serum thyroid hormone levels. However, in **Chapter 4** we demonstrate that these OATP1C1 polymorphisms are associated with fatigue and depression in a population of hypothyroid patients on adequate thyroid hormone replacement therapy. Although carriers of the OATP1C1-intron3C>T and the OATP1C1-C3035T polymorphism had less complaints of fatigue and depression, they showed no differences in neurocognitive functioning, nor did they appreciate LT4/LT3 combination therapy over normal LT4 replacement therapy. In **Chapter 5** we compared the four human members of the OATP1 subfamily. OATP1A2, OATP1B1, OATP1B3 and OATP1C1 transport (sulfated) iodothyronines and exhibit

overlapping substrate specificities. OATP1B1 and OATP1B3, both expressed exclusively in the liver, preferentially transport rT3 and iodothyronine sulfates, probably facilitating their degradation by liver D1. This reflects their role in the removal of endo- and xenobiotics from the body. OATP1C1 transports T4, T4S and rT3 across the blood-brain barrier, whereas OATP1A2 transports all (sulfated) iodothyronines.

Chapter 6 focused on the effect size of the TSHR-Asp727Glu polymorphism on serum thyroid parameters. Previously, this polymorphism has been associated with lower serum TSH levels. In a cohort of healthy Danish twins we determined the contribution of this polymorphism to overall variation in serum TSH levels. Although the TSHR-Asp727Glu polymorphism was significantly associated with serum TSH in this population as well, this polymorphism only explained 0.9% of the total variation in serum TSH levels. This suggests that multiple genes are involved in the control of hypothalamus-pituitary-thyroid axis. In **Chapter 7** this polymorphism was studied in relation to bone parameters, since TSH and thyroid hormone have independent effects on bone. In line with the effect of TSH on bone in mice, described by Abe and colleagues, serum TSH showed a positive trend with BMD in elderly Caucasians of the Rotterdam Study. Moreover, the TSHR-Asp727Glu polymorphism was associated with higher femoral neck BMD. However, serum FT4 had a much greater influence on bone than TSH.

Chapter 8 described studies performed with polymorphisms in D1. Previously, the D1-C785T and D1-A1814G polymorphisms, located in the 3'UTR of the *DIO1* gene, have been associated with serum thyroid hormone levels in several Caucasian populations. In this chapter we studied both the *in vivo* and *in vitro* effects of these polymorphisms. Although both polymorphisms were associated with rT3, and T3/rT3 ratio, no effect of both polymorphisms on D1 activity in transfected JEG3 cells was observed. In **Chapter 9** we focused on the relationship between thyroid function and blood pressure. Previously, TSH and FT4 have been associated with blood pressure. However, in two large cohorts of elderly Caucasians, no relationship between thyroid function and hypertension or blood pressure was observed. In addition, genetic variation in the hypothalamus-pituitary-thyroid axis has been implicated in blood pressure regulation and susceptibility to hypertension. However, in these two cohorts polymorphisms in D2 and the TSHR were not associated with blood pressure or the presence of hypertension. Therefore, we feel that thyroid function is not an important determinant of hypertension in elderly Dutch subjects.

Chapter 10 shows the results of a pathway analysis of genes involved in thyroid hormone synthesis, metabolism and transport using GWA data of the Rotterdam Study. Several polymorphisms in the *THRB*, *LRP2* and *SLCO1B1* genes were associated with serum TSH levels. In addition, polymorphisms in the *DIO1* and *SLC7A8* genes were associated with serum FT4 levels. Although the majority of these findings require replication, they are beginning to provide intriguing insights into the molecular basis of of population-based variation in serum TSH and FT4.

In the general discussion, **Chapter 11**, all results are discussed and put in a broader perspective. Methodological considerations, strengths and limitations of the research described in this thesis are discussed. Finally, the relevance and potential clinical implications are considered and suggestions for future research are made.

SAMENVATTING

De schildklier is een klein vlindervormig orgaan, dat zich bevindt zich voor het schildklierkraakbeen in de nek. Het belangrijkste product van de schildklier is schildklierhormoon. De schildklier produceert voornamelijk T4 (thyroxine), een inactieve vorm van schildklierhormoon. Door specifieke enzymen, de zogenaamde dejodases, kan T4 omgezet worden in het actieve schildklierhormoon T3. Het belangrijkste werkingsmechanisme van schildklierhormoon verloopt via binding van T3 aan de T3 receptor in de celkern. Zodra T3 bindt aan zijn receptor kunnen schildklierhormoon-gevoelige genen afgelezen worden. Hierdoor zullen bepaalde eiwitten aangemaakt worden of juist worden afgebroken. Aangezien de receptor zich in de celkern bevindt, hangt de biologische activiteit van schildklierhormoon af van de hoeveelheid hormoon in de bloedbaan en in de cel en van de aanwezigheid van dejodases en specifieke transporters, die schildklierhormoon vervoeren.

Schildklierhormoon is noodzakelijk voor de normale ontwikkeling en groei van vrijwel alle andere weefsels in ons lichaam. Te veel schildklierhormoon, dat wil zeggen hyperthyreoïdie, leidt tot gewichtsverlies, onrust, warmte-intolerantie en botontkalking. Hypothyreoïdie, te weinig schildklierhormoon, kan leiden tot moeheid, gewichtstoename, koude-intolerantie of zelfs depressiviteit. Minimale veranderingen van de spiegels van schildklierhormoon in het bloed kunnen belangrijke consequenties hebben. Zo kan iemand meer of minder vaatverkalking hebben, een lagere of snellere hartslag krijgen en tevens een hoger risico op depressie of botontkalking lopen. De studies, die beschreven worden in dit proefschrift, laten zien dat kleine veranderingen in genen, die betrokken zijn bij de productie, transport en verwerking van schildklierhormoon, hun invloed hebben op de bloedwaarden van schildklierhormoon. Tevens kunnen deze kleine genetische veranderingen leiden tot een verhoogd of juist verlaagd risico op botontkalking of het krijgen van een depressie. Een inleiding over de manier waarop schildklierhormoon gemaakt, verwerkt en getransporteerd wordt door het menselijk lichaam vindt u in **Hoofdstuk 1**. Tevens worden in dit hoofdstuk de onderzoeksvragen van dit proefschrift geformuleerd.

In **Hoofdstuk 2** is de rol van het eiwit OATP1B1 als schildklierhormoon transporter bestudeerd. Tevens hebben we het effect van het Val174Ala polymorfisme in het OATP1B1 gen bekeken. OATP1B1 vervoert geen T4 en T3, maar het is wel belangrijk voor het transport en de verwerking van bilirubine, E1S en gesulfateerd schildklierhormoon. Aangezien de OATP1B1-Ala^{174} variant minder transportactiviteit heeft, hebben dragers van het OATP1B1-Val174Ala polymorfisme tevens hogere bilirubine, E1S en T4S bloedwaarden dan niet-dragers. **Hoofdstuk 3** bespreekt de functie van OATP1C1, een eiwit dat ook onderdeel uitmaakt van de OATP1 familie. Het komt vrijwel alleen tot expressie op de bloed-hersen-barrière en vervoert T4, rT3 en in minder mate ook T4S. Sugiyama heeft aangetoond dat de expressie van OATP1c1 omhoog gaat in ratten met te weinig schildklierhormoon en omlaag gaat in ratten met hyperthyreoïdie. Op basis van zijn en onze gegevens denken we dat OATP1C1 belangrijk

is voor schildklierhormoontransport over de bloed-hersen-barrière. Genetische variaties in het OATP1C1 gen hebben geen relatie met schildklierhormoon spiegels in het bloed. Echter, in **Hoofdstuk 4** laten we zien dat deze polymorfismen wel een relatie hebben met vermoeidheid en depressie. In een groep patiënten met hypothyreoïdie, die behandeld worden met schildklierhormoon, hebben dragers van het OATP1C1-intron3C>T en het OATP1C1-C3035T polymorfisme minder klachten van vermoeidheid en depressiviteit. Er was, echter, geen verschil in neurocognitief functioneren of voorkeur voor T4/T3 combinatie boven T4 monotherapie. In **Hoofdstuk 5** hebben we de vier humane leden van de OATP1 familie vergeleken. OATP1A2, OATP1B1, OATP1B3 en OATP1C1 transporteren (gesulfateerd) schildklierhormoon en vertonen overlap wat betreft substraatspecificiteit. OATP1B1 en OATP1B3 komen beide alleen tot expressie in de lever. Ze transporteren alleen rT3 en gesulfateerd schildklierhormoon, waarna deze hormonen afgebroken worden door het type 1 dejodase in de lever. Dit weerspiegelt de rol van OATP1B1 en OATP1B3 in de verwijdering van afbraakproducten en lichaamsvreemde stoffen. OATP1C1 transporteert T4, T4S en rT3 over de bloed-hersenbarrière, terwijl OATP1A2 alle (gesulfateerde) schildklierhormonen transporteert.

In **Hoofdstuk 6** is de impact van het TSHR-Asp727Glu polymorfisme op schildklierhormoonspiegels in het bloed geanalyseerd. In eerdere studies is aangetoond dat dit polymorfisme geassocieerd is met lagere TSH waarden. In een groep van gezonde Deense tweelingen hebben we de bijdrage van dit polymorfisme aan de gehele variatie in TSH waarden bepaald. Hoewel het TSHR-Asp727Glu polymorfisme ook in deze populatie significant geassocieerd was met TSH, verklaarde deze variant slechts 0.9% van de totale variatie die er bestaat in TSH waarden. Dit suggereert dat er vele genen betrokken zijn in de controle van hypothalamus-hypofyse-schildklier as. In **Hoofdstuk 7** hebben we hetzelfde polymorfisme bestudeerd in relatie tot bot. TSH en schildklierhormoon hebben onafhankelijke effecten op bot. TSH is positief geassocieerd aan botmineraaldichtheid in een groep oudere Nederlanders. Dit is in overeenstemming met het effect van TSH op bot in muizen, voor het eerst beschreven door Abe en collega's. Ook het TSHR-Asp727Glu polymorfisme is geassocieerd met hogere bot mineraaldichtheid. Het effect van schildklierhormoon zelf op bot is echter veel groter dan dat van TSH.

Hoofdstuk 8 gaat over studies met betrekking tot polymorfismen in het type 1 dejodase. De D1-C785T en D1-A1814G polymorfismen zijn geassocieerd met schildklierhormoon spiegels in verschillende Caucasische populaties. In dit hoofdstuk hebben we de effecten van deze variaties op schildklierhormoon waarden in bloed en op de activiteit van type 1 dejodase in cellen bekeken. Hoewel beide polymorfismen wederom geassocieerd waren met rT3 en de T3/rT3 verhouding, zagen we geen effect van beide varianten op de activiteit van het type 1 dejodase in getransfecteerde JEG3 cellen. In **Hoofdstuk 9** hebben we de relatie tussen schildklierfunctie en bloeddruk onderzocht. In eerdere studies bleken zowel TSH als FT4 geassocieerd te zijn met bloeddruk. Echter, in twee grote populaties van oudere Nederlandse mannen en vrouwen konden wij geen associatie vinden tussen schildklierfunctie, bloeddruk

en/of verhoogde bloeddruk. Tevens hebben wij geen relatie gevonden tussen polymorfismen in het type 2 dejodase en de TSH receptor en bloeddruk, terwijl dit in een aantal andere studies wel naar voren kwam. Om deze reden concluderen wij dat in oudere Nederlanders de schildklierfunctie geen belangrijke rol speelt in het krijgen van een verhoogde bloeddruk

Hoofdstuk 10 beschrijft de resultaten van een analyse van genen die betrokken zijn bij de synthese, het metabolisme en het transport van schildklierhormoon. Met behulp van GWA data van het Rotterdam cohort hebben we polymofismen geïdentificeerd die geassocieerd zijn met TSH en FT4. Verschillende polymorfismen in het *THRB*, *LRP2* en *SLCO1B1* gen waren geassocieerd met serum TSH. Tevens waren polymorfismen in het *DIO1* en *SLC7A8* gen geassocieerd met serum FT4. De resultaten van deze studie moeten bevestigd worden in andere populaties. Echter, de rol van bepaalde genen, die betrokken zijn bij schildklierhormoon metabolisme, is wellicht toch anders dan in eerste instantie verondersteld. In de algemene discussie in **Hoofdstuk 11**, worden de resultaten besproken en in een breder perspectief geplaatst. Tevens worden de positieve en negatieve punten van het onderzoek besproken. Tenslotte worden de algemene klinische relevantie en toepasbaarheid van het onderzoek besproken en worden er aanbevelingen gedaan voor vervolgonderzoek.

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DANKWOORD

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About the author

ABOUT THE AUTHOR

Wendy Marianne van der Deure was born on October 11th, 1978 in Dordrecht. In 1997 she completed secondary school at the Insula Collega in Dordrecht, after which she started her medical study at the Erasmus University Rotterdam. During her study, she participated in an elective in internal medicine in Kumasi, Ghana for two months. Her graduation research in 2001 was performed at the Department of Hematology, University of Maryland in Baltimore, USA. She studied the effect of MS-275, a histone deacetylase inhibitor, on several leukemia cell lines. In September 2003 she obtained her medical degree. In 2004 Wendy worked as a resident in internal medicine at the Maasstad Hospital. In 2005 she started the work presented in this thesis at the department of internal medicine at the Erasmus Medical Center Rotterdam, under the enthusiastic supervision of Dr. R.P. Peeters and Prof. Dr. Ir. T.J. Visser. In August 2005 she received a travel grant from the European Thyroid Association based on her studies regarding the relationship between thyroid function and bone. For her work on the characterization of OATP1B1 as a thyroid hormone transporter she obtained a young investigator's travel grant award from the American Thyroid Association in June 2007. In April 2008 she received a travel grant from the Dutch Endocrine Society. In May 2008 she started her training residencies in internal medicine at the Maasstad Hospital in Rotterdam under the supervision of Dr. A. Berghout and Dr. M.A. van den Dorpel. On September 15, 2007 she married Jerry Gielisse.

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Abbreviations

ABBREVIATIONS

AN(C)OVA	analysis of (co)variance
BMI	body mass index
BMD	bone mineral density
bp	base pairs
BSA	bovine serum albumin
BSP	bromosulfophthalein
CI	confidence interval
CRYM	μ-crystallin
CVLT	California verbal learning test
D′	linkage disequilibrium coefficient
D1	type I deiodinase
D2	type II deiodinase
D3	type III deiodinase
df	degree of freedom
DNA	deoxyribonucleic acid
DZ	dizygotic
e.g	for example
E1S	estrone-sulfate
FT3	free T3
FT4	free T4
GWA	genome-wide association
HAT	heterodimeric amino acid transporter
HEZ	heterozygote
HOM	homozygote
HPLC	high performance liquid chromatography
HPT	hypothalamus-pituitary-thyroid
HWE	Hardy-Weinberg equilibrium
i.e.	in other words
INR	international normalized ratio
IRD	Inner ring deiodination
IU	international units
Kb	kilo base pairs
LAT	L-type amino acid transporter
LD	linkage disequilibrium
ln	natural logarithm
LT3	liothyronine
LT4	levothyroxine

MCT	monocarboxylate transporter
MCT	memory comparison test
MFI	multidimensional fatigue inventory
mRNA	messenger ribonucleic acid
MZ	monozygotic
NCBI	National Center for Biotechnology Information
NTCP	Na^+/taurocholate cotransporting polypeptide
OATP	organic anion transporting polypeptide
ORD	outer ring deiodination
ORF	open reading frame
OS	opposite sex
PASAT	paced auditory serial attention task
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
POMS	Profile of Moods States
PTU	6-propyl-2-thiouracil
RIA	radio-immuno assay
RT	reverse transcriptase
rT3	reverse T3
rT3S	reverse T3 sulfate
SD	standard deviation
SDS	sodium dodecyl sylfate
SECIS	selenocysteine insertion sequence
SEM	standard error of the mean
SCL	symptom checklist
SNP	single nucleotide polymorphism
SULT	sulfotransferase
T4	thyroxin (3,3′,5,5′-tetraiodothyronine)
T4S	T4 sulfate
T3	3,3′,5-triiodothyronine
T3S	T3 sulfate
TBG	T4-binding globulin
Tgabs	thyroglobulin antibodidies
TH	thyroid hormone
TPOabs	thyroid peroxidase antibodies
TRH	thyrotropin releasing hormone
TSH	thyroid-stimulating hormone (thyrotropin)
TSHR	thyroid-stimulating hormone receptor
TR	thyroid hormone receptor

TTR	transthyretin
3'UTR	3'untranslated region
3,3'-T2	3,3'-diiodothyronine
WHO	world health organization
WT	wild-type