# Regulation of Thyroid Hormone Bioactivity in Health and Disease

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# Regulation of Thyroid Hormone Bioactivity in Health and Disease

De regulatie van de bioactiviteit van schildklierhormoon in gezondheid en ziekte

## Proefschrift

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*voor Arzu*

## Contents

# *General Introduction*

Thyroid hormone plays a crucial role in a variety of metabolic and developmental processes in the human body. It is essential for growth and differentiation, for the regulation of energy metabolism, and for the physiological function of virtually all tissues (1, 2). It stimulates metabolic rate by increasing ATP turnover, and by regulating the expression of uncoupling proteins in the mitochondria of fat and skeletal muscle (3, 4). Decreased levels of thyroid hormone (hypothyroidism) result in a decreased metabolic rate and are associated with high levels of cholesterol, hypertension and atherosclerosis (2, 5-7). The importance of thyroid hormone for the development and function of the human central nervous system is illustrated by the fact that hypothyroidism during development results in severe mental retardation (8-10). Furthermore, even mild forms of maternal hypothyroidism during pregnancy are an independent determinant of a delay in infant neurodevelopment (11, 12). Increased levels of thyroid hormone (thyrotoxicosis) on the other hand result in an increased metabolic rate, weight loss, tachycardia, osteoporosis, and are associated with an increased risk of atrial fibrillation (2, 7, 13-16).

The production of thyroid hormone by the thyroid gland, in particular of the pro-hormone thyroxine (T4), is regulated by the classic hypothalamus-pituitary-thyroid axis (See Fig. 1) (2, 17). The biological activity of thyroid hormone, *i.e.*, the availability of the active hormone 3,5,3'-triiodothyronine (T3), is largely regulated by the iodothyronine deiodinases D1, D2 and D3 (2, 17, 18).

In the hypothalamus-pituitary-thyroid axis, the anterior pituitary releases thyroid stimulating hormone (TSH, thyrotropin) under the stimulation of thyrotropin releasing hormone (TRH), a tripeptide that is synthesized in the paraventricular nuclei of the hypothalamus (19, 20). TSH is the major factor regulating the synthesis and secretion of thyroid hormone by the thyroid. It mediates its effects via binding to the TSH receptor (TSHr), which is a G protein coupled receptor that acts via the protein kinase A and protein kinase C signalling pathways (21). TSH receptors are localized primarily at the basal surface of the cell (22). TSH stimulates the thyroid to produce the biologically inactive pro-hormone T4, which represents the majority of the hormone secreted by the thyroid. Most (~80%) of the circulating T3, the bioactive form of thyroid hormone, is therefore derived from conversion out of T4 in peripheral tissues, such as the liver. The biological activity of thyroid hormone is largely mediated via a mechanism of T3-regulated gene expression by specific nuclear T3 receptors in target tissues (23). Several thyroid hormone receptor (TR) isoforms exist, which are the products of the TRα and TRβ genes. Both T4 and T3 have an inhibitory effect on TRH and TSH secretion via a negative feed back loop mechanism.

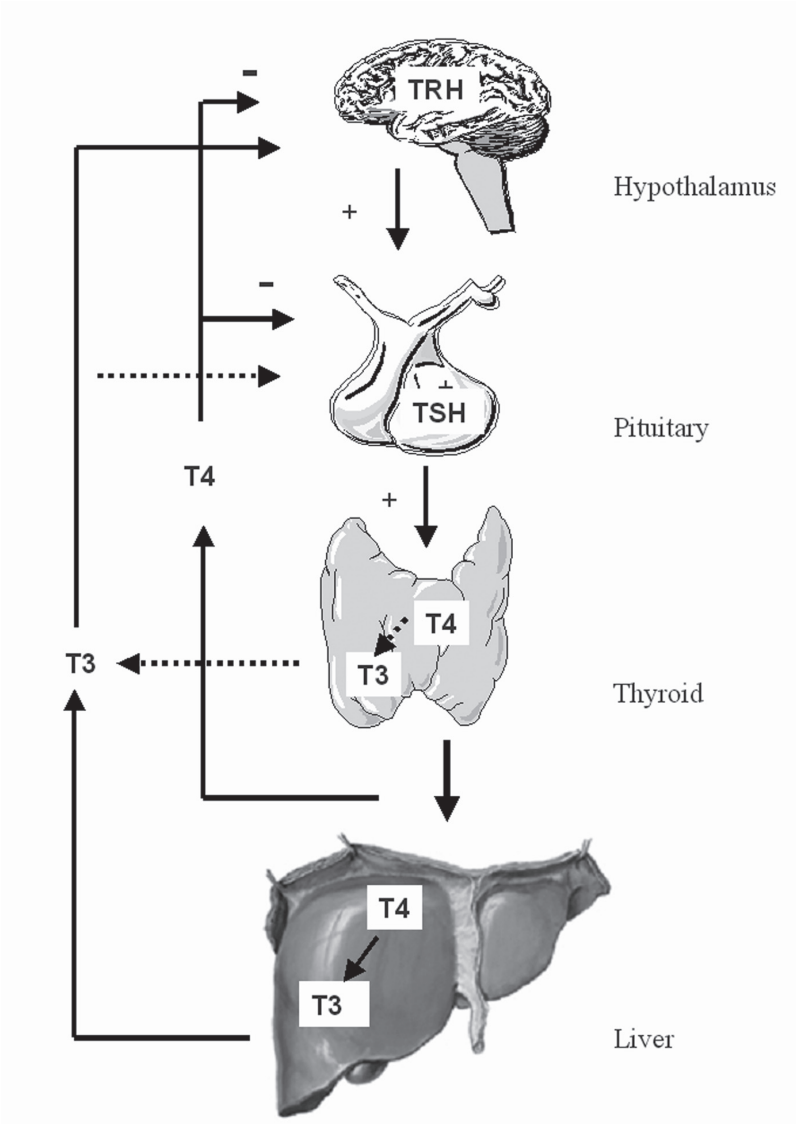


**Figure 1.** Simplified overview of the hypothalamus-pituitary-thyroid axis. Hypothalamic thyrotropin releasing hormone (TRH) stimulates the pituitary to release thyroid stimulating hormone (TSH). TSH regulates the production and secretion of T4 by the thyroid, which represents the majority of the hormone secreted by the thyroid. The majority of the circulating T3 is therefore derived from conversion out of T4 in peripheral tissues, illustrated in this figure by the liver, but other tissues, such as skeletal muscle, may also contribute to serum T3 production. Both T4 and T3 have an inhibitory effect on TRH and TSH secretion via a negative feed back loop mechanism.

Peripheral thyroid hormone metabolism is mediated importantly by the three deiodinases, that catalyze the inner- and outer ring deiodination of the different iodothyronines (see Refs. (17, 18) for reviews) (Figure 2, and Table 1). Outer ring deiodination is regarded as an activating pathway, whereas inner ring deiodination is an inactivating pathway.

D1 is present in liver, kidney, and thyroid, and plays a key-role in the production of serum T3 from T4 and in the breakdown of the metabolite rT3 (18). Deiodination of rT3 is the most efficient reaction catalyzed by D1. Although D1 has both outer ring and inner ring deiodination activities, its outer ring deiodination capacity is by far more important. The deiodination reaction catalyzed by D1 follows so-called ping-pong kinetics with two substrates. The first being the iodothyronine, the second being an endogenous intracellular thiol co-factor (24-27). The latter is then reduced by a yet unknown intracellular co-factor regenerating the enzyme. D1 is strongly inhibited by the thyrostatic drug 6-propyl-2-thiouracil (PTU) (28), which competes with the thiol cofactor and forms an irreversible dead-end complex with the enzyme. D1 activity is positively regulated by T3, which is due to the presence of two thyroid hormone response elements (TREs) in the 5'-flanking region of the gene. This positive regulation of D1 by T3 may explain why the plasma T3 production in hyperthyroid patients is much more sensitive to PTU than in euthyroid patients (29).

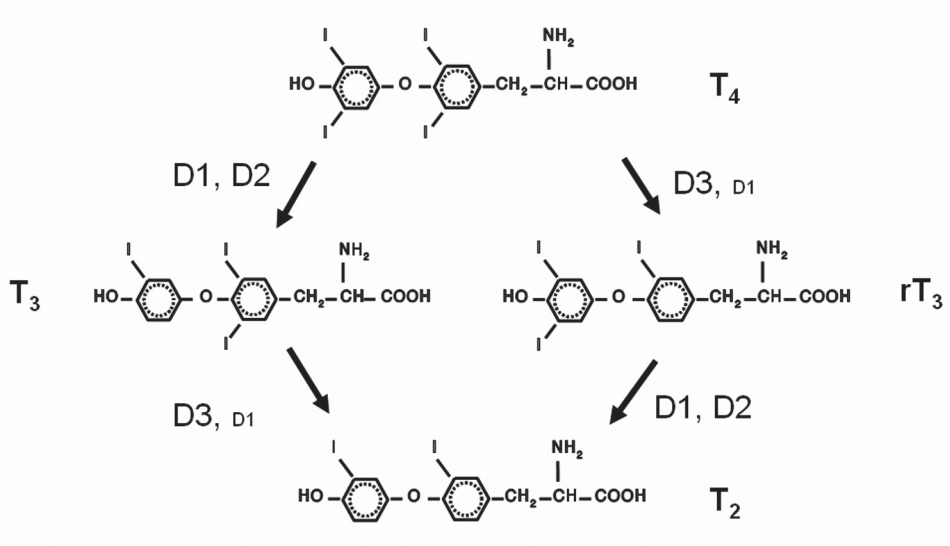


**Figure 2.** Structure and relationship between the different iodothyronines, and their activation and inactivation by the three deiodinases.

D2 is present in brain, anterior pituitary, brown adipose tissue (BAT), thyroid, and skeletal muscle, and D2 mRNA has also been detected in the human heart (18). D2 only has outer ring deiodination capacity and catalyzes the conversion of T4 to T3 and of rT3 to 3,3'-diiodothyronine (3,3'-T2). In tissues such as the brain, D2 is important for local production of T3, whereas D2 in skeletal muscle may also contribute to plasma T3 production (30, 31). Deiodination by D2 follows sequential reaction kinetics, which suggests that both the substrate and the thiol cofactor must combine with the enzyme before the reaction takes place (32). In contrast to D1, D2 is insensitive to PTU (32-34). In general, D2 activity is increased in hypothyroidism and decreased in hyperthyroidism. Both pre- and posttranslational mechanisms are involved in the regulation of D2 expression by thyroid state, with distinct roles for T3, and for T4 and rT3, respectively. T3 decreases D2 mRNA at the transcriptional level, without affecting the D2 mRNA half-life (35, 36), whereas T4 and rT3 decrease D2 activity by substrate induced ubiquitination and proteasomal degradation of the D2 protein (37, 38).

| | D1 | D2 | D3 |
|---|---|---|---|
| | T4 ↙ ↘ T3 rT3 ↘ ↙ T2 | T4 ↙ T3 rT3 ↙ T2 | T4 ↘ T3 rT3 ↘ T2 |
| Function | plasma T3 production, rT3 clearance | local and plasma T3 production | T3 and T4 clearance, rT3 production |
| Tissue distribution | Liver, kidney, thyroid | Brain, pituitary, BAT, thyroid, skeletal muscle, heart, aortic smooth muscle, osteoblasts | Brain, skin, placenta, fetal tissues, critically ill liver and skeletal muscle |
| Substrate preference | rT3>>T4=T3 | T4>rT3 | T3>T4 |

**Table 1.** Physiological role in thyroid hormone metabolism, tissue distribution, and substrate preference of the three different human iodothyronine selenodeiodinases (D1-D3).

D3 is present in brain, skin, placenta, pregnant uterus, and various fetal tissues (18). D3 has only inner ring deiodination activity, and catalyzes the inactivation of T4 and T3 by inner ring deiodination to rT3 and 3,3'-T2, respectively. It is the major T3 and T4 inactivating enzyme since D1 has only a weak inner ring deiodination capacity for nonsulfated T4 and T3. D3 shows preference for T3 over T4 as the substrate, and contributes to thyroid hormone homeostasis by protecting

tissues from excess thyroid hormone. In the fetal brain for example, D3 expression is highly regulated in different regions, protecting the brain from excessive T3 until differentiation is required (39). D3 activity is increased in hyperthyroidism and decreased in hypothyroidism, but the mechanism of this regulation remains to be established.

All three iodothyronine deiodinases are selenoproteins, and contain a single selenocysteine residue (SeC) in the catalytic center. This SeC is encoded by a UGA stop codon. A so-called SECIS (selenocysteine insertion sequence) element in the 3 'UTR of the deiodinases is the necessary signal that recodes this UGA stop codon into a SeC codon (40-42). The SeC residue is essential for enzymatic activity, and replacement with alanine or serine residues eliminates activity (43-45). Only replacement with a cysteine residue (substituting S for Se) either in D1, D2 or D3 maintains enzymatic activity, although with strongly reduced substrate turnover numbers and significantly increased $K_m$ values for the iodothyronine substrates (43-45).

The role of sulfation in thyroid hormone metabolism is intriguing. Sulfated iodothyronines do not bind to the thyroid hormone receptors, and sulfation mediates the rapid and irreversible degradation of iodothyronines by D1 (46). The serum concentrations of sulfated iodothyronines are therefore low in normal subjects (47-50). Inner ring deiodination of T4 and T3 by D1 is markedly accelerated after sulfation, whereas outer ring deiodination of T4 is blocked (51-53). D2 and D3 are incapable of catalyzing the deiodination of sulfated iodothyronines (52).

Since the deiodinases, as well as the T3 receptors, are located intracellularly, the metabolism and action of thyroid hormone require transport of iodothyronines across the cell membrane of target cells (54). Thyroid hormones are transported across the plasma membrane by carrier-mediated processes that are often found to be temperature, sodium, and energy dependent (55). Recently, several transporters capable of cellular uptake of iodothyronines have been identified (56). The most specific transporters identified so far are OATP1C1 and MCT8, which appear to be involved in T4 transport across the blood-brain barrier, and in T3 transport into brain neurons, respectively (57, 58). The MCT8 gene is located on the X chromosome, and inactivating mutations in MCT8 result in a phenotype of severe X-linked psychomotor retardation and elevated serum T3 levels (59).

## Genetic variation in thyroid hormone pathway genes

In healthy subjects, serum thyroid parameters show a substantial inter-individual variability, whereas the intra-individual variability is within a narrow range (60-64). This suggests an important influence of genetic variation and/or environmental

factors on the regulation of thyroid hormone bioactivity, which results in a unique thyroid function set-point for each individual (60, 62, 64). Recently published data from a classical twin study support this (65, 66). In this study, heritability accounted for ~64% of the variation in serum TSH, free T4 and free T3 concentrations, whereas environmental factors accounted for the other 36% (66). Furthermore, genetic factors accounted for ~71% of the individual differences in thyroid volume (65). In a Mexican-American population, total heritability in serum thyroid parameters ranged from 26–64% of the total inter-individual variation observed, whereas the effects of environmental covariates and a broad range of lifestyle covariates accounted for only 2–18% of the total phenotypic variation (67).

Polymorphisms are variations in the nucleotide sequence of the genome that occur in at least 1% of a population. Polymorphisms in thyroid hormone pathway genes may very well play an important role in the inter-individual variation in serum thyroid function tests, contributing to the unique set-point for each individual. DNA polymorphisms are stable markers and as such have an influence throughout a subject's life. These polymorphisms may therefore not only affect serum TSH and iodothyronine levels, but also result in an altered thyroid hormone bio-activity throughout life. Studies on subclinical hyper- and hypothyroidism illustrate that even minor alterations in thyroid hormone bio-activity may already have important consequences on thyroid hormone-related clinical endpoints such as atherosclerosis, bone mineral density, and heart rate (68, 69).

## Regulation of thyroid hormone bioactivity during critical illness

Pronounced alterations in plasma TSH and thyroid hormone levels occur during critical illness without any evidence for thyroid disease (70-73). Plasma T3 decreases and plasma rT3 increases within a few hours after the onset of disease, and the magnitude of these changes is related to the severity of the disease (18, 72, 73). In severely ill patients, also T4 decreases and both T4 and T3 are inversely correlated with mortality rate (72, 74). Despite a major decrease in T3, TSH levels remain within the low to normal range. The normal or low plasma TSH levels in critically ill patients, despite decreased T4 and T3 levels, suggest a major change in set point within the hypothalamus-pituitary-thyroid axis. The combination of decreased serum T3 and increased serum rT3 levels suggests that also major changes in the peripheral metabolism of thyroid hormone occur. An altered transport of thyroid hormone into deiodinase expressing tissues may also play a role (54).

Whether the reduction in serum T3 is a beneficial adaptation resulting in a decreased metabolic rate and a protection against hypercatabolism or whether it is

a mal-adaptation contributing to a worsening of the disease is still a controversial issue (70, 72, 75). So far, it has not been clearly demonstrated that substitution of critically ill patients with thyroid hormone has a positive or negative effect on clinical outcome (76-78). Intervention with hypothalamic releasing factors, which restores pulsatile pituitary hormone secretion, normalizes peripheral hormone levels, and keeps the negative feedback loop intact, might be a more successful approach (79-81).

## Outline of the thesis

The studies in this thesis consist of two major parts. In the first part of the thesis (**Part A**) we analyzed the genetic variation in thyroid hormone pathway genes. We identified severa l polymorphisms in thyroid hormone pathway genes and investigated if these polymorphisms contributed to the large inter-individual variability in serum thyroid parameters. We did so by analyzing if they were associated with serum TSH and iodothyronine levels in a healthy population of blood donors and in a population of elderly men (*Chapters A1*, *A2*, and *A3*). These polymorphisms may not only affect serum TSH and iodothyronine levels, but also result in an altered bio-activity of thyroid hormone throughout life. We therefore analyzed if they were associated with several thyroid hormone-related endpoints such as cholesterol metabolism (*Chapter A3*), growth hormone and IGF-I metabolism (*Chapter A2*), insulin resistance and body composition (*Chapters A2* and *A4*), and atrial fibrillation (*Chapter A5*).

In the second part of the thesis (**Part B**), we analyzed the regulation of thyroid hormone bio-activity during critical illness. We investigated if post-mortem deiodinase activities in liver and skeletal muscle biopsies are associated with peri-mortem serum thyroid parameters (*Chapters B1* and *B2*) and if serum TSH and iodothyronine levels are prognostic factors for survival (*Chapter B2*). Because of the decreased D1 expression during critical illness, and since D1 mediates the rapid and irreversible degradation of iodothyronine sulfates, we analyzed the metabolism of iodothyronine sulfates during critical illness in *Chapter B3* of this thesis. Since it is unclear if the changes in serum levels of thyroid parameters are also reflected by changes in tissue concentrations, we also investigated the regulation of local iodothyronine concentrations in liver and skeletal muscle (*Chapter B4*). In addition, we investigated cortisol metabolism in these patients (*Chapter B5*).

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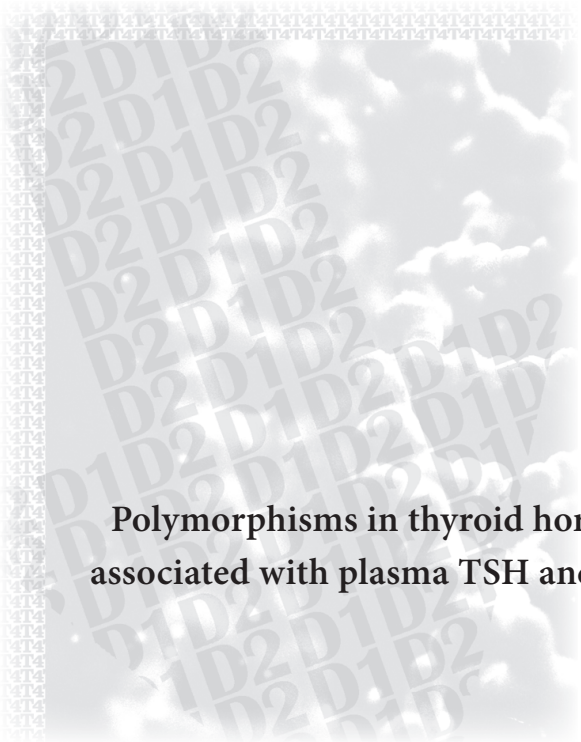
# *Part A*

## Genetic variation in thyroid hormone pathway genes



# *Chapter A1*

## Polymorphisms in thyroid hormone pathway genes are associated with plasma TSH and iodothyronine levels in healthy subjects

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## Abstract

**Introduction**: Single nucleotide polymorphisms (SNPs) in genes involved in thyroid hormone metabolism may affect thyroid hormone bioactivity. We investigated the occurrence and possible effects of SNPs in the deiodinases (D1-D3), the TSH receptor (TSHR), and the T3 receptor β (TRβ) genes.

**Methods:** SNPs were identified in public databases or by sequencing of genomic DNA from 15 randomly selected subjects (30 alleles). Genotypes for the identified SNPs were determined in 156 healthy blood donors and related to plasma T4, FT4, T3, rT3, and TSH levels.

**Results:** Eight SNPs of interest were identified, 4 of which have not yet been published. Three are located in the 3'-UTR: D1a-C/T (allele frequency C=66%, T=34%), D1b-A/G (A=89.7%, G=10.3%) and D3-T/G (T=85.5%, G=14.2%). Four are missense SNPs: D2-A/G (Thr92Ala, Thr=61.2%, Ala=38.8%), TSHRa-G/C (Asp36His, Asp=99.4%, His=0.6%), TSHRb-C/A (Pro52Thr, Pro=94.2%, Thr=5.8%) and TSHRc-C/G (Asp727Glu, Asp=90.7%, Glu=9.3%). One is a silent SNP: TRβ-T/C (T=96.8%, C=3.2%). D1a-T was associated in a dose-dependent manner with a higher plasma rT3 (CC 0.29±0.01; CT 0.32±0.01; TT 0.34±0.02 nmol/l (mean±SE), P=0.017), a higher plasma rT3/T4 (P=0.01), and a lower T3/rT3 (P=0.003) ratio. The D1b-G allele was associated with lower plasma rT3/T4 (P=0.024) and with higher T3/rT3 (P=0.08) ratios. TSHRc-G was associated with a lower plasma TSH (CC 1.38±0.07 vs. GC 1.06±0.14 mU/l, P=0.04), and with lower plasma TSH/FT4 (P=0.06), TSH/T3 (P=0.06), and TSH/T4 (P=0.08) ratios. No associations with TSH and iodothyronine levels were found for the other SNPs.

**Conclusion:** We have analyzed 8 SNPs in 5 thyroid hormone pathway genes and found significant associations of 3 SNPs in 2 genes (D1, TSHR) with plasma TSH or iodothyronine levels in a normal population.


## Introduction

Thyroid hormones play an essential role in a variety of metabolic and developmental processes in the human body. Most effects are mediated by the active thyroid hormone T3 via a mechanism of T3-regulated gene expression (1). Examples are the effects of thyroid hormone on brain development and skeletal maturation, on heat production and oxygen consumption, on the secretion and metabolic turnover of different hormones, and on the contractility of the heart (2-6). Production of thyroid hormone, in particular the pro-hormone T4, is controlled by the classic hypothalamic-pituitary-thyroid axis, while the biological activity of thyroid hormone,

*i.e.*, the availability of T3, is largely regulated by the iodothyronine deiodinases D1, D2 and D3 (4, 7, 8).

Polymorphisms (variations in the nucleotide sequences of the genome that occur in at least 1% of the population) in one or more genes involved in thyroid hormone metabolism may have subtle effects on thyroid hormone levels and thyroid hormone bioactivity throughout life. The symptoms of subclinical hyper- and hypothyroidism show that minor changes in thyroid hormone levels can have important consequences for quality of life, cognition, cholesterol metabolism, heart rate, bone mineral density, and atherosclerosis (9-14). For this reason we investigated genes encoding key proteins in thyroid hormone metabolism for the occurrence of single nucleotide polymorphisms (SNPs). The genes selected include the three selenodeiodinase genes D1, D2 and D3, the genes for the TSH receptor (TSHR), and the T3 receptor β (TRβ). These genes were screened for SNPs, preferentially those located in exons, by searching the public SNP database (dbSNP), the human expressed sequence tag database (dbEST), as well as the literature.

D1, D2, and D3 contain a selenocysteine (SeC) in their catalytic center, encoded by a UGA codon (15-17). In the vast majority of mRNAs, UGA is recognized as a stop codon. The incorporation of Sec into the deiodinases requires the presence of a SeC insertion sequence (SECIS) element in the 3'-UTR of the mRNA and several trans-acting factors (18). Because a SNP in this region may have important consequences for the production of functional deiodinase, the D1, D2, and D3 SECIS elements were sequenced in 15 randomly selected healthy subjects. Since little information is available in the databases about the possible polymorphisms in human D3, we also decided to analyze the coding sequence of the human D3 gene in these 15 subjects. A population of 156 healthy blood donors was genotyped with regard to these SNPs, and for each polymorphism the genotype was correlated with a specific set of plasma thyroid indexes.

For the SNPs in the three deiodinases, we analyzed correlations with plasma T4, T3, rT3 and with iodothyronine ratios. Plasma iodothyronine levels depend not only on the activities of iodothyronine-metabolizing enzymes but also - among other things - on thyroid function and plasma iodothyronine-binding capacity. Therefore, ratios between plasma iodothyronines are thought to better reflect tissue deiodinase activities. Local D2 activity is essential for the negative feedback regulation of hypophyseal TSH secretion by plasma T4, as demonstrated in the D2 knock-out mouse (19). Therefore, the relation between the D2 SNP and plasma TSH, and TSH/T4 and TSH/FT4 ratios was also analyzed. For the SNPs in the TSHR and in TRβ, we analyzed the correlation with plasma iodothyronines and TSH levels, and with the ratios thereof.

## Materials and Methods

### *Study population*

Blood was collected from 158 healthy anonymized blood donors at the Sanquin Blood Bank South West region (Rotterdam, The Netherlands). Informed consent was given by all donors. One subject was excluded because of plasma FT4 and TSH levels indicating hyperthyroidism, another because of hypothyroid FT4 and TSH levels. Gender was not documented for one subject. The study population thus consisted of 100 males and 55 females with an average age of 46.2 ± 12.2 yrs (mean±SD); 47.4 ± 10.9 yrs in the males, and 44.6 ± 13.9 yrs in the females. Donors on thyroid hormone treatment are not excluded from blood donation. Although ethnic background of donors has not been documented, over 99% may be of Caucasian origin. Descriptive statistics of this population are shown in Table 1.

**Table 1.** Descriptive statistics of the population analyzed in this study

| | Means ± SD (#) | Reference values ($) |
|---|---|---|
| N | 155 | |
| Age | 46.3 ± 12.2 *1) | |
| TSH (mU/l) | 1.32 ± 0.75 *2) | 0.4 - 4.3 |
| T4 (nmol/l) | 87.96 ± 15.9 | 58 - 128 |
| FT4 (pmol/l) | 15.06 ± 2.4 | 11 - 25 |
| T3 (nmol/l) | 1.96 ± 0.24 | 1.43 - 2.51 |
| rT3 (nmol/l) | 0.31 ± 0.08 | 0.14 - 0.34 |

(#) TSH and iodothyronine level of this population were determined in plasma.
($) Reference values used in our lab, with TSH and iodothyronine levels determined in serum.
*1) N = 156, *2) N = 154

### *Plasma analyses*

Plasma T4, FT4, T3 and TSH were measured by chemoluminescence assays (Vitros ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK). rT3 was measured by radioimmunoassay as previously described (20). TBG and anti-TPO antibodies were measured using chemoluminescence assays on an Immulite 2000 (D.P.C., Los Angeles, CA).

**Table 2.** Conditions and primers used for PCR amplification and subsequent sequencing of gDNA regions of interest

| | | PCR primers | Annealing Temperature | Sequencing primers |
|---|---|---|---|---|
| SECIS-D1 | Fw | 5'-TGAAATCTTCCACTAGCCTCA-3' | 50C | 5'-CAACAGAGTCATCTAGAAGGGA-3' |
| | Rv | 5'-ACTCCAGCAGGAGTTCATAAG-3' | | 5'-CACATTTAACATGTAACATAG-3' |
| SECIS-D2 | Fw | 5'-AAGCCAACAGGTAAACACATA-3' | 45C | 5'-CCAGGCCCATGTCACTGGTCA-3' |
| | Rv | 5'-AATACAAGAACAAATTGTATA-3' | | 5'-CACACATAGCACTCAGCACCA-3' |
| SECIS-D3 | Fw | 5'-AGTGCTTTGCCCGGTGCTTC-3' | 50C | 5'-GCTGCACAGAGACCTTGGCCA-3' |
| | Rv | 5'-CACCAAATGGCCTTCGAATCA-3' | | 5'-AATCAGCAGCTCTGCCTAGGA-3' |
| D3-CDS1 | Fw | 5'-CCCAGATGCCTCGCCAGGCCA-3' | 58C | 5'-TGGTCGGAGAGGGCGAGGGGTC-3' |
| | Rv | 5'-GCGCTCATGCGCGCCATGAA-3' | | 5'-TGCAGCTGCCGAAATTGAGAA-3' |
| D3-CDS2 | Fw | 5'-TTCTTCAAGCAGGCGCACGA-3' | 52C | 5'-CTCCGAGGTGGTTCTGCCCGA-3' |
| | Rv | 5'-CGCTTGCACGTGGGCTTCGAA-3' | | 5'-CTCGAAGGCCCAGCCCACCAA-3' |

**Table 3.** Conditions used for RFLP and SBE analysis (see materials and methods). Mismatches are shown by an underscore

| | | RFLP primers | Annealing Temp. | Restriction enzymes | | SBE primers |
|---|---|---|---|---|---|---|
| D1a-C/T | Fw | 5'-GAACTTGATGTGAAGGCTGGA-3' | 54C | Bcl I | Fw | 5'-($T_4$)GATACCTGAATTCTAGGTGA-3' |
| | Rv | 5'-TAACCTCAGCTGGGAGTTGTTT-3' | | | Rv | 5'-($T_4$)CTTGAGAAGCCCTCCCGTTG-3' |
| D1b-A/G | Fw | 5'-CAACAGAGTCATCTAGAAGGGA-3' | 48C | Spe I | Fw | 5'-($T_{10}$)TTATAAGATGCAGTAAACTA-3' |
| | Rv | 5'-CACATTTAACATGTAACATAG-3' | | | Rv | 5'-($T_{10}$)ATATTTTCATCATTCTGTTA-3' |
| D2-A/G | Fw | 5'-GATAGTAAAGAATAACAGCCTTGGCT-3' | 58C | Rsa I | Fw | 5'-($T_{16}$)GTGTGGTGCATGTCTCCAGT-3' |
| | Rv | 5'-CAGCTATCTTCTCCTGGATACCA-3' | | | Rv | 5'-($T_{16}$)ACTGTTGTCACCTCCTTCTG-3' |
| D3-T/G | Fw | 5'-CTGGTAGGGGAAGTGATCTCG-3' | 60C | BssSI | Fw | 5'-($T_{22}$)TGGTAGGGGAAGTGATGTCG-3' |
| | Rv | 5'-GCCAATGCCTCTCAAGCTATC-3' | | | Rv | 5'-($T_{22}$)CTGCCCACCCCTCCCCATCC-3' |
| TSHa-G/C | Fw | 5'-ATTTCGGAGGATGGAGAAATA-3' | 53C | Bsp 1286 I | | |
| | Rv | 5'-GTCTGCGTACTGGGCGGTAA-3' | | | | |
| TSHb-C/A | Fw | 5'-GCGATTTCGGAGGATGGAGAAATAGC-3' | 58C | Tth 111 I | Fw | 5'-($T_{28}$)AACGCATCCCCAGCTTACCG-3' |
| | Rv | 5'-CCGGGTACTCACAGAGTCTGCGACCTG-3' | | | Rv | 5'-($T_{28}$)CTTCAGAGTCTGCGTACTGG-3' |
| TSHc-C/G | Fw | 5'-AACGCCAGGCTCAGGCATAC-3' | 60C | Nla III | Fw | 5'-($T_{34}$)GTTCAAAAGGTTACCCACGA-3' |
| | Rv | 5'-AAGTTCCCCTACCATTGTGA-3' | | | Rv | 5'-($T_{34}$)TTGTGGAGACCCTGCCTCAT-3' |
| TRß-T/C | | | | | Fw | 5'-($T_{40}$)AAACACCACGTGACACACTT-3' |
| | | | | | Rv | 5'-($T_{40}$)TTCATCAGGAGTTTAGGCCA-3' |

### *DNA isolation*

DNA was extracted from 2 ml of blood using the PUREGENE™ DNA Isolation Kit (Gentra Systems, Minneapolis, MN) with slight modifications of the provided protocol. After isolation, DNA concentration was measured at 260 nm, and all samples were diluted to a concentration of 50 ng/l (stock) and 10 ng/µl (work solution). Purity was determined by measuring the 260/280 nm ratio.

### *Sequence analysis*

DNA from 15 random subjects was used for sequence analysis. The SECIS-element and flanking regions were analysed in the D1, D2, and D3 genes. At first, DNA was amplified by polymerase chain reaction (PCR) using the primers listed in Table 2. All primers used in this study were ordered from Invitrogen (Breda, The Netherlands). The PCR was performed in a GeneAmp® PCR system 9700 Thermocycler (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) and conditions were as follows: 5 min at 96 C; 35 cycles of 1 min at 94 C, 1 min at annealing temperature (Table 2) , and 1 min at 72 C; and finally 7 min at 72 C. PCR products were verified by agarose gel electrophoresis. Subsequently, they were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Almere, The Netherlands). To increase specificity, sequencing of the PCR products was performed using internal primers (Table 2). Samples were purified using Micro Bio-Spin P-30 Tris columns (Bio-Rad, Veenendaal, The Netherlands), and sequenced directly on an automated ABI 310 capillary sequencer (Applied Biosystems), using the Big Dye Terminator Cycle Sequencing method (Applied Biosystems).

### *Database searches*

Nucleotide sequences of the genes of interest were obtained from NCBI (http://www.ncbi.nlm.nih.gov). The TBLASTN program was used to search the EST database (dbEST) for sequences that differ one or more nucleotides from the wild-type (WT) gene. To exclude sequencing artefacts, at least two ESTs with the same nucleotide difference were required, before this variation was marked as a potential SNP. In addition, the SNP databases of NCBI (dbSNP) was searched.

### *Restriction fragment length polymorphism (RFLP) analysis*

PCR-RFLP procedures were developed for the D1a-C/T, D1b-A/G, D2-Thr/Ala, D3-T/G, TSHRa-Asp/His, TSHRb-Pro/Thr, and TSHRc-Asp/Glu polymorphisms. The primers used are listed in Table 3. For D2-A/G, a mismatch primer was used to

destroy a second restriction site for RsaI. For D3-T/G and TSHRb-C/A, mismatch primers were used to generate restriction sites for BssSI and Tth 111 I, respectively. The RFLP for TSHRb-C/A was described previously (21). Twenty ng of genomic DNA was amplified in a PCR reaction with a total volume of 10 μL. The PCR mixture contained 1x PCR buffer (Invitrogen), 0.2 mM of each dNTP, 1.5 mM MgCl, 2 pmol of each primer, and 0.5 U Taq polymerase (Invitrogen). Annealing temperatures of the different PCRs are listed in Table 3. Five units of restriction enzyme were used for a 1 h digestion of the PCR product at the recommended temperature. Table 3 lists the restriction enzymes used for RFLP analysis of the different SNPs. Digestion products were analyzed by agarose gel electrophoresis. All subjects were genotyped for the D1a-C/T, D1b-A/G, D2-Thr/Ala, D3-T/G, TSHRa-Asp/His, TSHRb-Pro/Thr, and TSHRc-Asp/Glu polymorphisms using RFLP. Examples are presented in Figure 2.

### *Single Base Extension (SBE) analysis*

To validate the genotypes, two independent methods were used. In addition to RFLP, genotyping was also performed by SBE analysis. All subjects were genotyped for the D1a-C/T, D1b-A/G, D2-Thr/Ala, D3-T/G, TSHRb-Pro/Thr, TSHRc-Asp/Glu, and TRβ-T/C polymorphisms using SBE. PCR products were generated using the same primers as used for RFLP analysis (Table 3), except for TSHRb and TRβ. A PCR fragment of TSHR1b was generated using 5'-ATTTCGGAGGATGGAGAAATA-3' as the forward primer, and 5'-GCAGATGCCCTTGATCTCTG-3' as the reverse primer. Annealing temperature was 58 C. For TRβ1, 5'-TGCCTGTGTTGAGAGA-ATAG-3' was used as the forward primer and 5'-GTCTAATCCTCGAACACTTC-3' as the reverse primer; annealing temperature was 51 C. All other conditions were the same as mentioned above for the other PCRs. The SBE reactions were performed using the ABI Prism® SNaPshot™ ddNTP Primer Extension Kit (Applied Biosystems) with slight modifications of the protocol provided by the manufacturer. For SBE analysis of the D1a, D3, and TSHRb SNPs the reverse primers were used, and for the other SNPs the forward primers were used.

### *Functional characterization of D2-Thr92Ala variant*

A human D2 expression vector (pcDNA3-hD2-rSECIS), containing the human D2 cDNA inserted 5' to the SECIS element of the rat D1 gene, was used as template for site-directed mutagenesis via the circular mutagenesis procedure (22, 23). Overlapping sense and antisense primers containing the nucleotide changes needed to produce the Thr92Ala D2 mutant (sense 5'-GTGCATGTCTCCAGTGCAGAAGGAG-

GTGACAAC) cDNA were used in circular mutagenesis reactions with 50 ng plasmid template and 2 U of Pfu DNA polymerase (23). Plasmid DNA isolated from 5 clones was sequenced directly on an automated ABI 310 capillary sequencer to verify that the desired mutation had been generated, and that no spurious mutations had occurred during amplification.

The WT and variant D2 enzymes were expressed in COS-cells (65cm$^2$ dishes) by DEAE-dextran-mediated transfection with 8 µg plasmid DNA (22). After two days, the cells were rinsed with PBS and collected in 0.25 ml PE buffer (0.1 M phosphate (pH=7.2), 2 mM EDTA), sonicated, aliquoted, and stored at – 80 C.

The principle of the *in vitro* D2 activity assay is the production of radioiodide by outer-ring deiodination of [3',5'-$^{125}$I]T4 or [3',5'-$^{125}$I]rT3. Duplicate incubations contained about 100,000 cpm labeled T4 or rT3 with varying amounts of unlabeled substrate (T4 or rT3) and COS cell homogenates (50-100 µg protein) in a total volume of 0.2 ml of PED20 buffer (20 mM DTT). Mixtures were incubated for 60 min at 37 C, where after the production of radioiodide was determined as described (22).

The principle of the *in situ* D2 activity assay is the release of [$^{125}$I]T3 in the medium of D2 expressing COS-1 cells incubated with [3',5'-$^{125}$I]T4. COS cells were cultured in 6-wells plates and transfected with 2.5 µg plasmid per well as described (22). One day after transfection, cell monolayers were washed with serum-free DMEM/F12 medium and then cultured for an additional 24 h in serum-free DMEM/F12 (plus 40 nM selenite), to which were added [$^{125}$I]T4 (10 $^6$ cpm/ml) and 1-1000 nM unlabeled T4. Medium was harvested, extracted and analyzed by HPLC as described (22).

### *Statistical analysis*

Data were analysed using SPSS 10.0.7 for Windows (SPSS Inc., Chicago, IL). Logarithmic transformations were applied to normalize variables and to minimize the influence of outliers if applicable. Differences in plasma thyroid hormone levels between the genotype groups were adjusted for age and sex and tested by ANCOVA using the general linear model procedure. Results are reported as mean ± SE in the figures, and as mean ± SD in the tables. Comparison of the frequencies of the genotypes between males and females was carried out using a Chi-square test. Deviation from Hardy-Weinberg equilibrium was also analyzed using a Chi-square test. P values are two-sided throughout, and $P<0.05$ was considered significant. Haplotypes allele frequencies were estimated using the computer program 3LOCUS.pas (24).

## Results

### *Identification of SNPS*

Figure 1 shows an overview of the different SNPs identified. Previous unpublished studies in our laboratory had shown a variation at nucleotide position 785 of the D1 cDNA sequence (GenBank identification GI 4557521) which is occupied by a C or a T. This polymorphism was confirmed in the dbEST, and is referred to as D1a-C/T (Fig. 1). Sequencing of the region around the SECIS element of the D1 gene showed an A/G variation in 2 of the 15 subjects at position 1814 (D1b-A/G), 33 nucleotides downstream of the SECIS element. This variation was confirmed in the EST database. No other SNPs in D1 were found in at least 2 different databases.

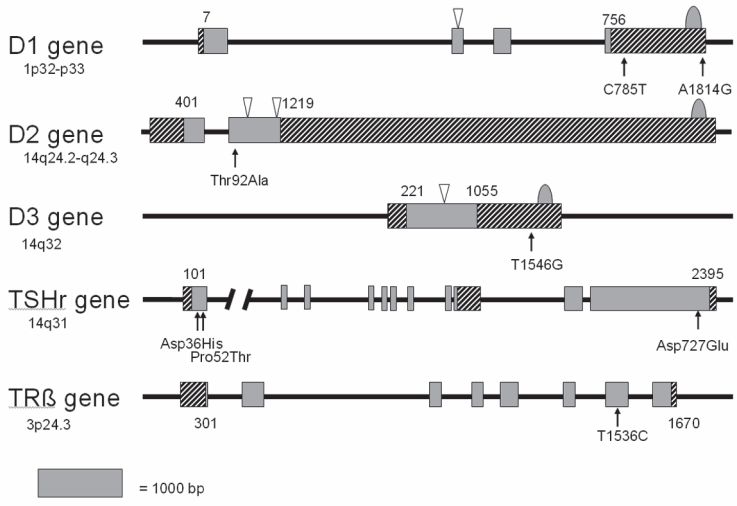


**Figure 1.** The thyroid hormone pathway genes analyzed in this study and in which polymorphisms were identified. Nucleotide numbers are based on the following Genbank accession numbers: 4557521 for D1, 13654872 for D2, 4503334 for D3, 4507700 for TSHR, and 10835122 for TRß. Size of the different exons is indicated by their scale. The coding sequence is represented by ▭, whereas ▨ indicates the untranslated region. An UGA codon, representing a selenocysteine, is depicted by ▽. Finally, SECIS elements are indicated by ◠.

No variation was detected by sequencing of the region around the SECIS element of the D2 gene. However, screening of the dbEST showed a very frequent A/G polymorphism at nucleotide 674 of the D2 sequence (GI 13654872), predicting a change in amino acid 92 of the protein (D2-G/A; Thr92Ala) (Fig. 1).

Sequencing of the coding sequence of the D3 gene showed no variation in the 15 healthy subjects. However, a T/G polymorphism was found at position 1546 of

the D3 cDNA sequence (GI 4503334), 66 nucleotides upstream of the SECIS element (D3-T/G) in 2 of the 15 subjects. This polymorphism was confirmed in the dbSNP.

Screening of the literature revealed three SNPs in the coding sequence of the TSHR gene. The Asp36His variation (TSHRa-G/C polymorphism in nucleotide 206 of the TSHR cDNA sequence (GI 4507700)), and the Pro52Thr variation (TSHRb-C/A polymorphism in nucleotide 254) are located in the extracellular domain of the receptor, whereas the Asp727Glu variation (TSHRc-C/G polymorphism in nucleotide 2281) is situated in the intracellular domain. TSHRb-C/A and TSHRc-C/G were also identified in the dbSNP.

A silent T/C polymorphism in TRβ gene (TRβ-T/C), corresponding to position 1536 of the TRβ1 cDNA sequence (GI 10835122), and to amino acid 412 (Phe) of the TRβ1 protein, was found in previous studies in our laboratory (F Wassen, unpublished results).

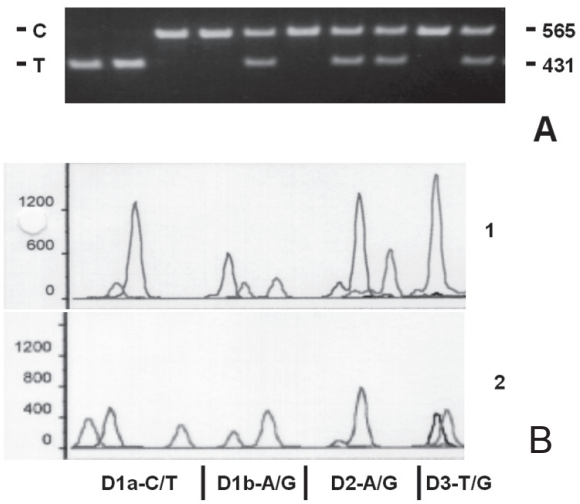


**Figure 2A.** RFLP analysis of 10 subjects for D1a-C/T. A PCR fragment was generated of 565 base pairs (bp). Incubation with BcI I generates two fragments of 434 and 131 bp only in the presence of the D1a-T allele (only the 565 and 434bp fragment are shown in this figure).
**Figure 2B.** SBE analysis of 2 subjects for D1a-C/T, D1b-A/G, D2-A/G, D3-T/G. Orange peaks represent the size marker, blue peaks represent a C, green peaks represent a T, and a black peak represents a G. The reverse primer was used for D1b-A/G and D2-A/G (see materials and methods). By developing primers of a different size, it is possible to analyze several SNPs in one SBE reaction. Subject 1 is heterozygous for D1b-A/G and D2-A/G, whereas subject 2 is heterozygous for D1a-C/T and D3-T/G.

Figure 2 shows examples of 1 RFLP analysis and of 2 SBE analyses. All SBE and RFLP data were concordant, with the restriction that we could not make a clear distinction by RFLP between heterozygous D3-GT and homozygous D3-GG subjects. Frequencies of the different genotypes and alleles are depicted in Table 4. Except for TSHRa-Asp36His, all SNPs were frequent enough to perform statistical analysis. Except for TSHRb-Pro52Thr, all distributions were in Hardy-Weinberg equilibrium (Table 4).

**Table 4.** Frequencies of genotypes and alleles for the polymorphisms identified.

| Polymorphism | Genotype frequencies | | | Allele frequency | HWE *P* |
|---|---|---|---|---|---|
| | CC | CT | TT | C=66% | |
| D1a-C/T | 65 (41.7%) | 76 (48.7%) | 15 (9.6%) | T=34% | 0.28 |
| | AA | AG | GG | A=89.7% | |
| D1b-A/G | 124 (79.5%) | 32 (20.5%) | 0 | G=10.3% | 0.15 |
| | AA | AG | GG | A=61.2% | |
| D2-A/G | 58 (37.2%) | 75 (48.1%) | 23 (14.7%) | G=38.8% | 0.88 |
| | TT | TG | GG | T=85.5% | |
| D3-T/G | 112 (72.3%) | 42 (27.1%) | 1 (0.6%) | G=14.2% | 0.16 |
| | GG | GC | CC | G=99.4% | |
| TSHRa-G/C | 154 (98.7%) | 2 (1.3%) | 0 | C=0.6% | 0.94 |
| | CC | CA | AA | C=94.2% | |
| TSHRb-C/A | 139 (89.7%) | 14 (9.0%) | 2 (1.3%) | A=5.8% | 0.03* |
| | CC | CG | GG | C=90.7% | |
| TSHRc-C/G | 127 (81.4%) | 29 (18.6%) | 0 | G=9.3% | 0.20 |
| | TT | TC | CC | T=96.8% | |
| TRß-T/C | 145 (93.5%) | 10 (6.5%) | 0 | C=3.2% | 0.68 |

Except for TSHRb-C/A, distribution of all genotypes was in Hardy-Weinberg equilibrium (HWE).

### *Association of SNPs with plasma TSH and iodothyronine levels*

D1a is a very frequent polymorphism. The T-allele of D1a was associated in a dose-dependent manner with increasing plasma rT3 levels: CC 0.29±0.01; CT 0.32±0.01; TT 0.34±0.02 nmol/l, (mean ± SE, P=0.017, corresponding to 18.8, 20.8, and 22.1 µg/dl), (Fig. 3A). The difference corresponds to a 33% of SD increase per allele copy. In addition, we observed the T-allele to be associated with increasing plasma rT3/

T4 ratio (P=0.01, Fig 3B), and decreasing plasma T3/rT3 ratio (P=0.003). The G allele of the D1b-A/G polymorphism was less frequent and was associated with lower plasma rT3/T4 (P=0.024) and higher T3/rT3 (P=0.08) ratios. Due to the low frequency of the polymorphism, the difference in rT3 levels failed to reach significance (P=0.17) (Fig. 3C and 3D).

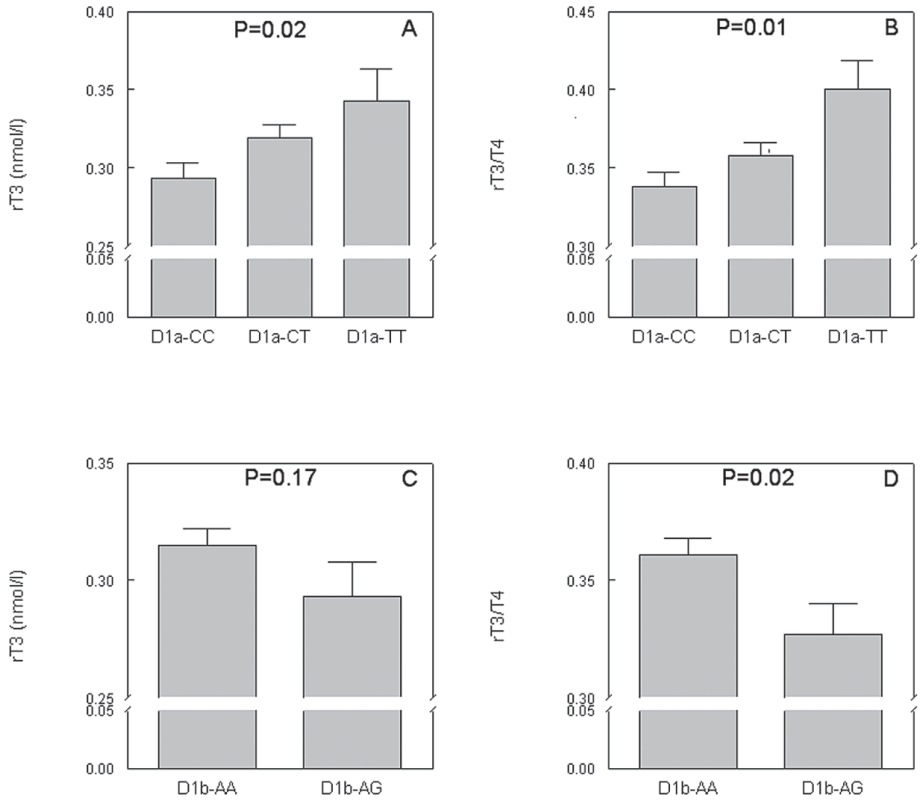


**Figure 3.** Differences between plasma rT3 concentrations (A & C) and plasma rT3/T4 ratios (B & D) by genotype for the D1a (A&B) and D1b (C & D) polymorphism. P values represent an ANOVA test (C) or an ANOVA test for linearity (A, B). To convert values for rT3 to ng/dl, divide by 0.0154.

These two D1 SNPs were analyzed for linkage disequilibrium, and haplotype allele frequencies were calculated. The results revealed that there were only 3 different haplotype alleles in this population (1 = aC-bA, 2 = aT-bA, 3 = aC-bG, with a frequency of 0.56, 0.34, and 0.10, respectively). Based on these three haplotype alleles, all individuals could be genotyped and the effect of the individual alleles on plasma thyroid hormone parameters could be assessed. The haplotype 1 showed no correlation with rT3, rT3/T4 or with T3/rT3 ratio. The haplotype 2 showed a positive correlation with rT3 (P=0.017) and rT3/T4 ratio (P=0.01), and a negative relation with T3/rT3 ratio (P=0.003) (Fig. 4A and 4B). Due to the low frequency of the haplotype allele 3, statistical analysis had limited power. A negative relation with rT3 (P=0.172) and with rT3/T4 (P=0.024), and a positive relation with T3/rT3 (P=0.08) was observed.

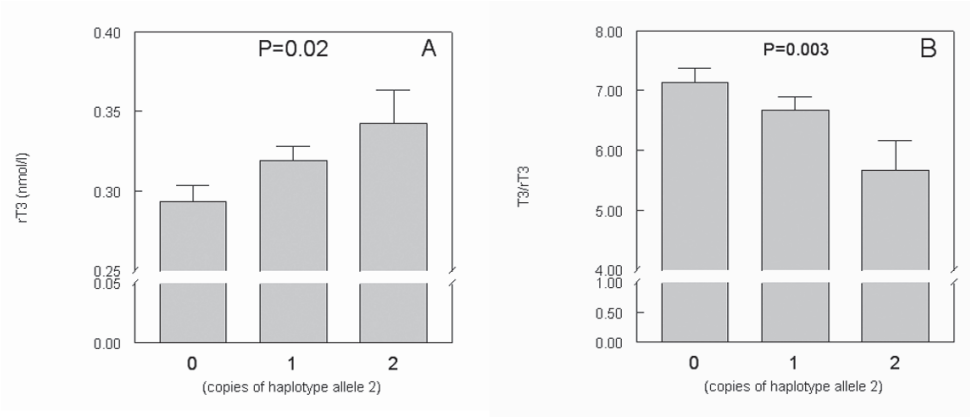


**Figure 4.** Differences in rT3 levels (A) and T3/rT3 ratios (B) in subjects analyzed by genotype for haplotype alleles for the combined D1a and D1b polymorphisms (1=C-A, 2=T-A, 3=C-G). P values represent an ANOVA test for linearity. To convert values for rT3 to ng/dl, divide by 0.0154.

D2-A/G was the most frequent polymorphism in this study. No significant correlation was found with plasma iodothyronine levels in this population. However, plasma TSH levels were lower in subjects heterozygous for this D2 polymorphism (GA 1.17 ± 0.08 vs. AA 1.44 ± 0.11 and GG 1.49 ± 0.13 mU/l, P=0.07). Also, the plasma TSH/T4 ratio was lower in heterozygous subjects (P=0.08).

For the D3-T/G polymorphism the frequency of genotypes was 72.3% TT, 27.1% TG, and 0.6% GG. No significant correlation between this polymorphism and plasma iodothyronines was observed.

Only 2 subjects in this study possessed the TSHRa-His variant. Therefore, statistical power was limited to analyze the relation with plasma levels for this polymorphism. No associations were found for the TSHRb-Pro52Thr polymorphism with plasma iodothyronine or TSH levels in this population. For the TSHRc-Asp727Glu polymorphism, heterozygous subjects showed decreased TSH levels (P=0.04, Fig. 5), and tended to have lower plasma TSH/FT4 (P=0.06), TSH/T4 (P=0.08), and TSH/T3 (P=0.06) ratios than subjects homozygous for the Asp allele.

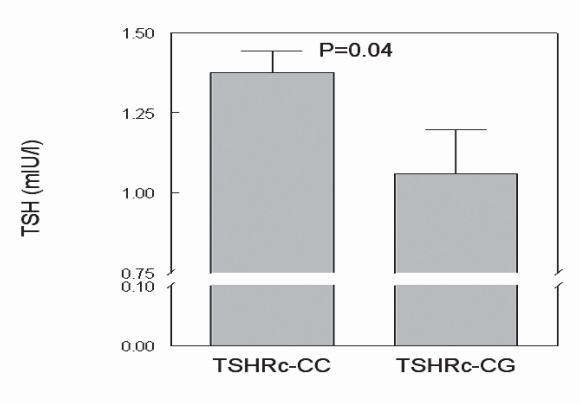


**Figure 5.** Difference in plasma TSH concentration between subjects homozygous for the TSHRc-Asp variant and subjects heterozygous for the TSHRc-Asp/Glu variant (P<0.05).

No association was found for the TRβ-T/C polymorphism with plasma iodothyronine and TSH levels in our population.

*Functional characterization of D2-Thr92Ala variant*

To define the effects of the substitution of the Thr residue at position 92 in D2 by Ala, expression vectors were made encoding WT-D2 and D2-Thr92Ala enzyme. Homogenates of cells, transfected with WT-D2, D2-Thr92Ala, or a 1:1 mixture of WT-D2 and D2-Thr92Ala enzymes, displayed similar Km and Vmax values, using T4 or rT3 as substrate (Table 5, Figure 6A). Upon incubation of intact transfected cells with increasing amounts of unlabeled T4, the fractional deiodination of [$^{125}$I]T4 decreased in a similar way (Figure 6B). No differences in deiodination of T4 were observed between cells expressing WT-D2, D2-Thr92Ala, or a combination of both.

**Table 5**. Kinetic characteristics of D2 enzymes (incubations in presence of 20 mM DTT)

| Deiodinase | $K_m$ (T4) nM | $V_{max}$ (T4) pmol T4/min.mg | $V_{max}$ / $K_m$ | $K_m$ (rT3) nM | $V_{max}$ (rT3) pmol rT3/min.mg | $V_{max}$ / $K_m$ |
|---|---|---|---|---|---|---|
| D2-WT | 4.3 3.1* | 0.32 0.61* | 0.07 0.20* | 17 | 0.34 | 0.02 |
| D2-Thr92Ala | 5.5 3.1* | 0.49 0.69* | 0.09 0.22* | 19 | 0.38 | 0.02 |

* Results of two separate experiments are shown, using T4 as a substrate

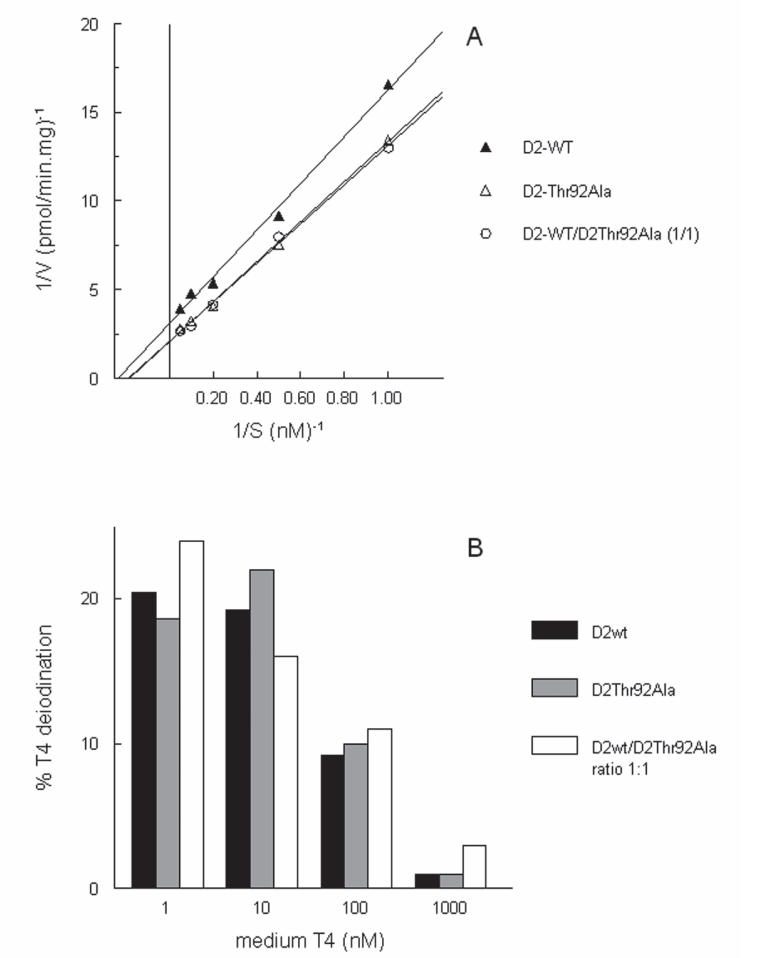


**Figure 6A.** Lineweaver-Burk plot of T4 deiodination by D2-WT, D2-Thr92Ala, or D2-WT/D2Thr92Ala (1/1) at 20mM DTT in the presence of varying concentrations of T4.
**Figure 6B.** *In situ* deiodination at varying T4 concentrations. COS cells were transfected with D2-WT, D2-Thr92Ala, or D2-WT/D2Thr92Ala (1/1) expression vectors, and intact cell deiodination was determined as described in *materials and methods*.

## Discussion

To date, little information has been available regarding the presence of functionally relevant polymorphisms in thyroid hormone pathway genes. In this study we focused on the D1, D2, D3, TSHR, and TRβ genes and identified 8 polymorphisms of interest. Four of them (D2-Thr92Ala, TSHRa-Asp36His, TSHRb-Pro52Thr, and TSHRc-Asp727Glu) have been reported previously (21,25-27). This is the first study that reports the polymorphisms D1a-C/T, D1b-A/G, D3-T/G, and TRβ-T/C. Three are located in the 3'-UTR of the deiodinase genes, *i.e.*, D1a-C/T, D1b-A/G and D3-T/G, while one, TRβ-T/C, is a silent SNP. No other polymorphisms have been reported in any of the three deiodinases so far.

We found a significant correlation between the D1a-C/T polymorphism and plasma rT3, with rT3 levels progressively increasing in subjects with zero, one, or two D1a-T alleles. The T variant was also positively correlated with the plasma rT3/T4 ratio, and negatively correlated with the T3/rT3 ratio. Although the D1b-A/G polymorphism was not significantly correlated with plasma rT3, the G variant was negatively correlated with the rT3/T4 ratio and positively with the T3/rT3 ratio. Liver D1 plays a key-role in the production of plasma T3 from T4 and in the breakdown of the metabolite rT3 (7, 8). RT3 is by far the preferred substrate for D1 (7, 8). For this reason, a functionally relevant SNP in D1 is expected to affect plasma iodothyronine levels, in particular rT3, and ratios between plasma iodothyronines (7, 8). These data suggest a negative effect of the D1a-T variant on total D1 activity, whereas the D1b-G variant appears to have a positive effect. This is supported by the haplotype analysis, which showed a negative relation of the C-G haplotype allele (haplotype 3) and a positive relation of the T-A haplotype allele (haplotype 2) with plasma rT3 and rT3/T4 levels. These haplotype alleles had an opposite effect on plasma T3/rT3 levels. Since both SNPs are located in the 3'-UTR, a change in the stability of the mRNA is an attractive explanation. Another explanation may be an altered folding of the mRNA, which - at least at the site of the SECIS element - is necessary for the incorporation of the selenocysteine. Alternatively, the association could be explained by linkage disequilibrium with another SNP located in the coding sequence or in regulatory areas of the gene such as the promoter region of the D1 gene. However, in this study we did not find any evidence for SNPs in the coding sequence of D1.

We did not see a significant correlation of the D1a and D1b SNPs with plasma T3 concentration, which can be explained by the fact that there are many pathways for T3 metabolism, such as glucuronidation, sulfation, and deiodination by D1 and D3. In addition, there are three sources of plasma T3, *i.e.*, thyroidal T3 secretion,

outer ring deiodination (ORD) of T4 by D1, and ORD of T4 by D2 (7, 28). On the other hand, rT3 turnover is less complicated; it is largely produced by inner ring deiodination of T4 by D3, and cleared by D1-catalyzed ORD. This may explain why no correlation was found of the SNPs in D1 with plasma T3, whereas we did find a significant correlation with rT3 levels.

Local D2 activity is essential for the negative feedback regulation of hypophyseal TSH secretion by plasma T4, as demonstrated in the D2 knock-out mouse (19). No significant effect of the D2-Thr92Ala polymorphism was seen on plasma TSH levels, iodothyronine levels or their ratios in this healthy population. However, D2 is particularly important for local T3 production in D2-containing tissues (7, 8), and based on this study, it can not be excluded that intracellular thyroid hormone levels are affected by this polymorphism.

The D2-Ala92 variant was previously reported to be associated with insulin resistance (25). However, the functional consequences of this polymorphism with regard to deiodinase activity were not investigated in this study (25). To determine the possible functional effects of this polymorphism, we performed *in vitro* analysis of the D2-Thr92Ala variant by transfection of COS cells. In an attempt to mimic the heterozygous situation, cells were also transfected with a combination of both variants. No significant differences in D2 activity were observed, neither when assays were done on homogenates in the presence of excess DTT as cofactor, nor in intact cells in the presence of the enzyme's natural cofactor. Whether the previously reported association (25) can be explained by linkage with another polymorphism, and whether this association can be confirmed in different populations, should be explored in future studies.

D3 is present in human brain, placenta, skin and in various fetal tissues, and is the major T4 and T3-inactivating enzyme by catalyzing their conversion to rT3 and 3.3'-T2, respectively. It plays an essential role during fetal development where it protects the embryo from excess thyroid hormone (29). We did not find any effect of the D3-T/G polymorphism on plasma iodothyronine levels in this population. The effects of this polymorphism on tissue thyroid hormone levels, and, thus, for instance on brain development and function, should be explored in future studies.

The growth and function of the thyroid is controlled by TSH through its receptor (30). Many mutations in TSHR have been identified as causes of thyroid diseases (30, 31). In addition to these mutations, three interesting polymorphisms in the coding sequence of the TSHR gene have been described. TSHRa-Asp36His and TSHRb-Pro52Thr polymorphisms are situated in the extracellular domain of the receptor, whereas the TSHRc-Asp727Glu polymorphism is located in the

intracellular domain. The His variant of the TSHRa-Asp36His polymorphism was detected in only 2 subjects in our population, indicating an allelic frequency of <1%, which is not significantly different from the frequency reported by Gustavsson *et al*. (26). No further statistical analysis was performed for this polymorphism.

The TSHRb-Pro52Thr polymorphism was more frequent, with an allelic frequency of the Thr variant of 6%, which is in agreement with other studies (21, 32). The genotype distribution of this SNP is not in Hardy-Weinberg equilibrium, which may be explained by the small size of the groups or by multiple testing, rather than by population bias. Conflicting observations regarding the response of this variant to TSH stimulation (33-35) could reflect the subtle effects of this polymorphism. We found no association of this polymorphism with plasma TSH levels, which can be due to the low frequency of the SNP. This SNP should therefore be analyzed in future studies in larger populations.

The allele frequency of the Glu variant of the TSHRc-Asp727Glu polymorphism is 9.5%, in agreement with results reported by Noguiera *et al*. (36). Conflicting data have been obtained regarding the cAMP response to TSH of this variant receptor in transfection studies (36, 37). No evidence was found for an association of the TSHRc-Asp727Glu polymorphism with non-autoimmune hyperfunctioning thyroid disorders (27). However, we found a significant association of the TSHRc-727Glu variant with lower plasma TSH levels and, although not significant, with lower plasma TSH/T4, and TSH/FT4 ratios. These data agree with findings showing an increased cAMP response of the TSHRc-Glu variant (37) to TSH, since less TSH would be required to achieve a normal thyroid hormone production. Together these data suggest that this is a functionally relevant SNP.

We identified a silent polymorphism in the coding sequence of the TRβ gene (T1536C), with an allelic frequency of the C variant of 3%. No evidence was found in our population for an association of the TRβ-C/T polymorphism with plasma TSH or iodothyronine levels, or their ratios. Due to the low number of subjects with the TRβ-T variant, no conclusions can be drawn from our observations regarding the (lack of) functional importance of this polymorphism.

In conclusion, we have analyzed the possible association of 8 SNPs in 5 thyroid hormone pathway genes with plasma TSH and iodothyronine levels. Four of those polymorphisms have not been described before. We find significant effects for 3 SNPs on plasma TSH and/or thyroid hormone levels. Due to the low frequency of several other SNPs, no conclusions can be drawn in our current population about their effect on plasma TSH and/or thyroid hormone levels.

## Acknowledgements

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# *Chapter A2*

## A polymorphism in type I deiodinase is associated with circulating free IGF-I levels and body composition in humans

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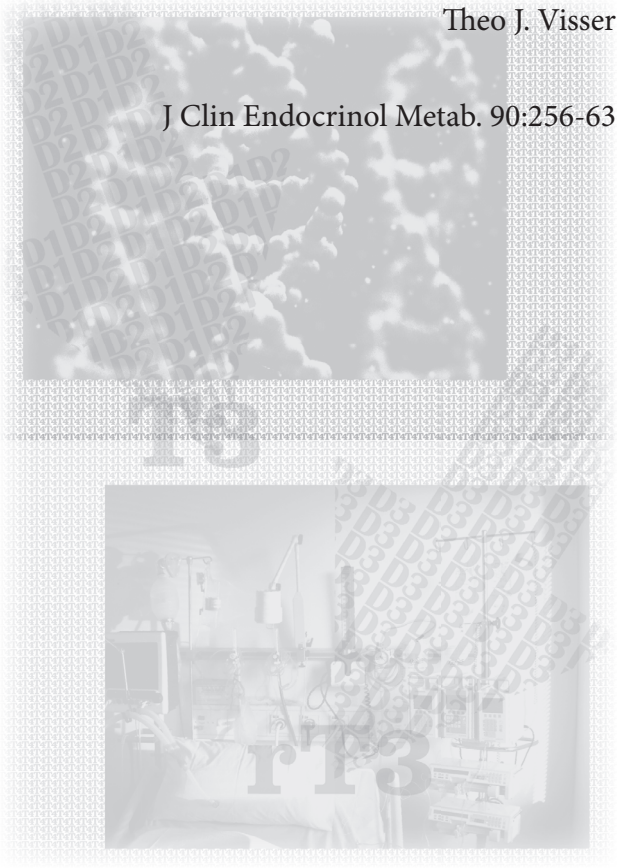
J Clin Endocrinol Metab. 90:256-63



## Abstract

**Introduction:** The interaction between the GH-IGF-I axis and thyroid hormone metabolism is complex and not fully understood. T4 stimulates IGF-I activity in animals in the absence of GH. On the other hand, GH replacement therapy results in an increase in serum T3 and a decrease in T4 and rT3 levels, suggesting a stimulation of type I deiodinase (D1) activity. Recently, we demonstrated the association of two polymorphisms in D1 (D1a-C/T; T=34%, and D1b-A/G; G=10%) with serum iodothyronine levels. Haplotype alleles were constructed, suggesting a lower activity of the D1 haplotype 2 allele (aT-bA) and a higher activity of the haplotype allele 3 (aC-bG). In this study, we investigated if genetic variations in D1 are associated with the IGF-I system.

**Methods:** In 156 blood donors and 350 elderly men, the association of the D1 haplotype alleles with circulating IGF-I and free IGF-I levels was studied. In addition, potential associations with muscle strength and body composition were investigated in the elderly population. Finally, the relation between serum iodothyronine levels and IGF-I levels was studied.

**Results:** In blood donors, haplotype allele 2 was associated with higher levels of free IGF-I (302.9 ± 22.9 vs. 376.3 ± 19.1, pg/ml, P=0.02). In elderly men, haplotype allele 2 also showed an allele dose increase in free IGF-I levels ($P_{trend}$=0.01), and an allele dose decrease in serum $T_3$ levels ($P_{trend}$=0.01), independent of age. Carriers of the D1a-T variant also had a higher isometric grip strength (P=0.047) and maximum leg extensor strength (P=0.07), as well as a higher lean body mass (P=0.03). In blood donors, T4 and FT4 were negatively correlated with total IGF-I levels (R=-0.18, P=0.03 and R=-0.24, P=0.003), whereas T3/T4 and T3/rT3 ratios were positively correlated with total IGF-I (R=0.31, P<0.001 and R=0.18, P=0.03). Free IGF-I showed a negative correlation with T4 (R=-0.26, P=0.001), and TBG (R=-0.31, P<0.001), and a positive correlation with T3/T4 ratio (R=0.21, P0.01).

**Conclusion:** A polymorphism that results in a decreased D1 activity is associated with an increase in free IGF-I levels. The pathophysiological significance of this association with IGF-I is supported by an increased muscle strength and muscle mass in carriers of the D1 haplotype 2 allele in a population of elderly men. The association of D1 haplotype allele 2 with serum T3 levels in the elderly population suggests a relative increase in its contribution to circulating T3 in old age.

## Introduction

The interaction between the growth hormone (GH)-IGF-I axis and thyroid hormone metabolism is complex and not fully understood. The anabolic effects of growth hormone are mediated by IGF-I. Free IGF-I by analogy with sex and adrenal steroids and thyroid hormones, may be the major biologically active hormonal form of IGF-I (1). GH replacement therapy in GH-deficient subjects acutely increases serum T3 levels, with a decrease in serum T4 and rT3 (2-5). This suggests a stimulatory effect of GH and/or IGF-I on the activity of type I deiodinase (D1), the enzyme mainly responsible for the production of serum T3 from T4 and for the clearance of rT3 (6). D2 in skeletal muscle may also contribute to serum T3 production (6).

On the other hand, euthyroidism is essential for normal growth and development of many tissues (7-9). The effects of thyroid hormone on growth have been explained by its ability to promote the secretion of GH, since it is required for a normal GH expression, both in vitro (10-12) and in vivo (13). However, not all effects of thyroid hormone on the IGF-I system are mediated by GH, since thyroid hormone itself also interacts with IGF-I (14-16). Treatment of hypophysectomized or thyroidectomized rats with T4 results in a stimulation of IGF-I activity in the absence of GH (14, 15), and the administration of GH to hypothyroid rats or humans fails to reverse growth impairment unless T4 is administered concurrently (16).

Previous studies have reported various alterations of the GH-IGF-I axis during hyper- and hypothyroidism. Hypothyroid adults are reported to have low concentrations of IGF-I (17-20), with a restoration to normal after euthyroidism is restored. Hyperthyroid patients are reported to have normal (21, 22) or high levels of IGF-I (18, 23, 24) (25). Normalization of thyroid function results in a decrease in IGF-I (18, 24, 25), or does not alter IGF-I (21). However, free IGF-I levels do not alter during hyperthyroidism (25).

Recently, we observed effects of two genetic single nucleotide polymorphisms (SNPs) in the D1 gene (D1a-C/T and D1b-A/G) on plasma thyroid hormone levels, suggesting a lower D1 activity in subjects with the D1a-T variant and a higher D1 activity in subjects with the D1b-G variant (26). In this study, we investigated the relation of D1 polymorphisms with circulating IGF-I levels, as well as with body composition and muscle strength in elderly men. Furthermore, we investigated the correlation between serum thyroid hormone levels, and both total and free IGF-I levels in healthy subjects. The ratios of the different iodothyronines were also analyzed, since these ratios better reflect peripheral thyroid hormone metabolism.

## Materials and Methods

### *Study populations*

#### *Healthy blood donors*

Blood was collected from 158 healthy anonymized blood donors at the Sanquin Blood Bank South West Region (Rotterdam, The Netherlands) (26). Informed consent was given by all donors. One subject was excluded because of serum FT4 and TSH levels indicating hyperthyroidism, another because of hypothyroid FT4 and TSH levels. Gender was not documented for one subject. The mean age of the study population was 46.2 ± 12.2 years (mean±SD) (47.4 ± 10.9 years in the males (N=100), and 44.6 ± 13.9 years in the females (N=55)). Donors on thyroid hormone treatment were not excluded from blood donation. Descriptive statistics of this population are shown in Table 1a. DNA was extracted from 2 ml of blood using the PUREGENE™ DNA Isolation Kit (Gentra Systems, Minneapolis, MN) with slight modifications of the provided protocol. After isolation, DNA concentration was measured at 260 nm, and all samples were diluted to a concentration of 50 ng/µl (stock) and 10 ng/µl (work solution). Purity was determined by measuring the 260/280 nm ratio.

#### *Healthy elderly men*

DNA was available of 350 subjects of a cross-sectional, single-center study in 403 independently living men, 70 years of age and higher (27). In this study, names and addresses of all male inhabitants 70 years and older were drawn from the municipal register of Zoetermeer, a medium sized town in the Midwestern part of The Netherlands. A total of 1567 men were invited, and after exclusion of subjects who did not live independently and subjects who were not physically or mentally able to visit the study center independently, eventually 403 men participated (25.7%). Participants signed an informed consent. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam. See reference (27) for a more detailed description of this population. 26 subjects in this population were not of Caucasian origin, the majority coming from Indonesia. Height and weight were measured in standing position without shoes. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters. Furthermore, lean body mass and fat mass were measured by dual-energy x-ray absorptiometry (DEXA) (28, 29). Quality assurance for DEXA, including calibration, was performed every morning, using the standard provided by the manufacturer.

**Table 1a.** Characteristics of the population of healthy blood donors

| | N | Mean | SD | Minimum | Maximum |
|---|---|---|---|---|---|
| AGE (yrs) | 156 | 46 | 12 | 19 | 69 |
| TSH (μU/mL) | 154 | 1.32 | 0.75 | 0.02 | 5.46 |
| T4 (μg/dL) | 155 | 6.84 | 1.24 | 4.47 | 12.35 |
| FT4 (ng/dL) | 155 | 1.17 | 0.19 | 0.81 | 1.82 |
| T3 (ng/dL) | 155 | 127.7 | 15.9 | 97.6 | 188.1 |
| RT3 (ng/dL) | 155 | 20.2 | 5.4 | 9.1 | 34.5 |
| TBG (mg/dL) | 155 | 1.83 | 0.43 | 1.14 | 4.09 |
| Total IGF-I (ng/mL) | 154 | 200.4 | 99.4 | 36.0 | 579.0 |
| Free IGF-I (pg/mL) | 154 | 345.7 | 198.1 | 18.4 | 987.5 |

All hormone measurements were performed in EDTA plasma. To convert T4 from μg/dL to nmol/L and FT4 from ng/dL to pmol/L, multiply by 12.87. To convert T3 and rT3 from ng/dL to nmol/L, multiply by 0.0154. To convert Total IGF-I from ng/mL to nmol/L and Free IGF-I from pg/mL to pmol/mL, multiply by 0.1307.

Isometric grip strength (IGS) was measured using an adjustable hand held dynamometer (JAMAR dynamometer) at the non-dominant hand (30). Each test was repeated three times, and the average was used in the analyses. Leg or knee extensor strength (LES) was measured as described previously (31), using the Hoggan MicroFET hand-held dynamometer. To obtain one main outcome measurement for LES, "maximum LES" (maxLES) was defined as the maximum strength for the right or left leg, whichever is largest, in a position of 120-degree extension. Statistical analyses were based on the physical unit momentum (Nm), obtained by multiplying the maximum strength (in Newton) and the distance of the dynamometer to the knee joint (in meters). See Table 1b for the descriptive of this population. Five subjects out of the 350 received thyroid hormone replacement therapy, 28 of them had diabetes.

**Table 1b.** Characteristics of the population of 350 elderly men

| | N | Mean | SD | Minimum | Maximum |
|---|---|---|---|---|---|
| AGE (yrs) | 350 | 77.7 | 3.5 | 73 | 94 |
| TSH (μU/mL) | 338 | 1.15 | 0.92 | 0.09 | 9.43 |
| T4 (μg/dL) | 337 | 6.28 | 1.17 | 3.65 | 10.02 |
| FT4 (ng/dL) | 338 | 1.29 | 0.24 | 0.64 | 2.45 |
| T3 (ng/dL) | 338 | 93.7 | 14.5 | 54.0 | 135.4 |
| RT3 (ng/dL) | 338 | 21.6 | 5.8 | 9.8 | 48.2 |
| TBG (mg/dL) | 338 | 2.73 | 0.69 | 0.60 | 6.00 |
| Total IGF-I (ng/mL) | 350 | 769.5 | 222.6 | 239.4 | 1500.0 |
| Free IGF-I (pg/mL) | 350 | 685.4 | 410.0 | 152.2 | 4038.7 |
| BMI (kg/m2) | 350 | 25.3 | 3.0 | 15.9 | 34.9 |
| WHR | 349 | 0.98 | 0.05 | 0.85 | 1.14 |
| Total Fat Mass (kg) | 350 | 20.9 | 5.5 | 3.6 | 39.0 |
| Lean Body Mass (kg) | 350 | 51.6 | 5.7 | 33.1 | 73.6 |
| IGS (kp) | 350 | 34.4 | 6.9 | 14.7 | 56.0 |
| Maximum LES (Nm) | 350 | 102.9 | 20.8 | 32.0 | 145.2 |

All hormone measurements were performed in serum. To convert T4 from μg/dL to nmol/L and FT4 from ng/dL to pmol/L, multiply by 12.87. To convert T3 and rT3 from ng/dL to nmol/L, multiply by 0.0154. To convert Total IGF-I from ng/mL to nmol/L and Free IGF-I from pg/mL to pmol/mL, multiply by 0.1307.

## *Serum and plasma analyses*

### *Healthy blood donors*

In this population, all hormone measurements were performed in EDTA plasma. T4, FT4, T3 and TSH were measured by chemoluminescence assays (Vitros ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK). rT3 was measured by RIA as previously described (32). TBG was measured using chemoluminescence assays on an Immulite 2000 (D.P.C., Los Angeles, CA). Plasma free IGF-I was measured with a commercially available, noncompetitive, two-site immunoradiometric assay (Diagnostic Systems Laboratories, Veghel, The Nether-

lands) (33). Plasma total IGF-I was measured by an IGFBP-blocked RIA (Medgenix Diagnostics, Fleurus, Belgium), as described previously (34); the intra-assay and interassay coefficients of variation were below 11%.

### *Healthy elderly men*

Blood samples were collected in the morning after an overnight fast. Serum was separated by centrifugation and stored at –40 C. TSH was measured using an immunometric technique (Amerlite TSH-30, Ortho-Clinical Diagnostics). Serum T4, T3, and rT3 were all measured by RIA, FT4 by Amerlite MAB FT4 assay (Ortho-Clinical Diagnostics). TBG was also measured by RIA. Intra- and interassay variability coefficients of all the assays were below 11%. Free IGF-I and total IGF-I were measured similar as described for the blood bank donors.

## *Genotyping*

### *Healthy blood donors*

PCR-RFLP procedures were developed for the D1a-C785T and D1b-A1814G polymorphisms (26), (see also Fig 1). The primers used are listed in Table 2. Twenty ng of genomic DNA was amplified in a PCR reaction with a total volume of 10 μL. The PCR mixture contained 1x PCR buffer (Invitrogen), 0.2 mM of each dNTP, 1.5 mM MgCl, 2 pmol of each primer, and 0.5 U Taq polymerase (Invitrogen, Breda, The Netherlands). Annealing temperatures of the different PCRs are listed in Table 2. Five units of restriction enzyme were used for a 1 h digestion of the PCR product at the recommended temperature. Table 2 lists the restriction enzymes used for RFLP analysis of the two polymorphisms. Digestion products were analyzed by agarose gel electrophoresis. All subjects were genotyped for the D1a-C/T and D1b-A/G polymorphisms using RFLP.

The genotypes, determined by RFLP, were confirmed by Single Base Extension (SBE) analysis (26). PCR products were generated using the same primers as used for RFLP analysis (Table 2) and using the same conditions as described above. The SBE reactions were performed using the ABI Prism® SNaPshot™ ddNTP Primer Extension Kit (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) with slight modifications of the protocol provided by the manufacturer. For SBE analysis of the D1a polymorphism, the reverse primer was used, and for the D1b polymorphism the forward primer was used.

**Table 2.** Conditions used for RFLP and SBE analysis (see materials and methods).

| | | | Annealing Temperature | Restriction enzymes |
|---|---|---|---|---|
| | | ***RFLP primers*** | | |
| D1a-C/T | Fw | 5'-GAACTTGATGTGAAGGCTGGA-3' | 54C | Bcl I |
| | Rv | 5'-TAACCTCAGCTGGGAGTTGTTT-3' | | |
| D1b-A/G | Fw | 5'-CAACAGAGTCATCTAGAAGGGA-3' | 48C | Spe I |
| | Rv | 5'-CACATTTAACATGTAACATAG-3' | | |
| | | ***SBE primers*** | | |
| D1a-C/T | Fw | 5'-($T_4$)GATACCTGAATTCTAGGTGA-3' | | |
| | Rv | 5'-($T_4$)CTTGAGAAGCCCTCCCGTTG-3' | | |
| D1b-A/G | Fw | 5'-($T_{10}$)TTATAAGATGCAGTAAACTA-3' | | |
| | Rv | 5'-($T_{10}$)ATATTTTCATCATTCTGTTA-3' | | |

Mismatches are shown by an underscore

### *Healthy elderly men*

All subjects were genotyped for the two polymorphisms using only SBE as described above.

### *Statistical analysis*

Data were analyzed using SPSS 10.0.7 for Windows (SPSS Inc., Chicago, IL). Logarithmic transformations were applied to normalize variables and to minimize the influence of outliers if applicable. Differences between the genotype groups were adjusted for age, sex and TBG levels if appropriate and tested by ANCOVA using the general linear model procedure. Pearson's correlation coefficients were used to calculate correlations between IGF-I and thyroid hormone metabolites after correction for age, and if necessary for sex and, or TBG. The effects of the polymorphisms on serum indices of thyroid function were analyzed after the exclusion of the subjects on thyroid hormone treatment (n=5). The age-dependent changes in thyroid hormone levels are often determined or accompanied by a poor health status, and there is an increased prevalence of non-thyroidal illness in the elderly (35, 36). For this reason, the association of the polymorphisms with serum thyroid hormone levels was re-analyzed after the exclusion of all subjects with non-thyroidal illness (T3 < 88 and rT3 > 20.8 ng/dL, n=53). Because of the interaction of diabetes and antidiabetic drugs with IGF-I metabolism, the effect of the different polymorphisms on IGF-I metabolism was analyzed after the exclusion of all subjects with diabetes

(n=28). Results are reported as mean ± SE in the figures, and as mean ± SD in the tables. Deviation from Hardy-Weinberg equilibrium (HWE) was analyzed using a Chi-square test. All genotype distributions were in HWE. Frequencies of the genotypes were the same in the Caucasian and the non-Caucasian population. P- values are two-sided throughout, and P<0.05 was considered significant. Haplotype allele frequencies were estimated using the computer program 3LOCUS.pas (37).

## Results

### *Association of SNPs in D1 with serum and plasma levels*

#### *Healthy blood donors*

Previous haplotype analysis of the D1a and D1b polymorphisms revealed only three different haplotype alleles in this population (26) (1 = aC-bA, 2 = aT-bA, 3 = aC-bG), with a frequency of 0.56, 0.34, and 0.10 respectively.) (Fig. 1). We demonstrated that the haplotype 2 allele was associated in a dose-dependent manner with higher rT3 and lower T3/rT3 levels, suggesting a lower D1 activity in subjects with this haplotype allele (26). Haplotype allele 3 showed an opposite relation. In order to increase statistical power, subjects that were heterozygous and homozygous for a specific haplotype allele were combined as carriers and analyzed *versus* subjects without this allele (non-carriers). The haplotype allele 2 showed a significant, positive relation with free IGF-I levels. Mean free IGF-I levels were 73.4 pg/mL lower in subjects without allele 2 compared to subjects with allele 2 (302.9 ± 22.9 vs. 376.3 ± 19.1, P=0.02, 95% CI –133.1 to -13.8) (Fig. 2). Adjustment for the effect of haplotype allele 2 on plasma thyroid hormone levels, or for TBG levels, did not alter the effect of this haplotype allele on circulating free IGF-I levels. No effect of allele 2 was observed on total IGF-I levels.

Due to the lower frequency of the D1b polymorphism, no subjects homozygous for haplotype allele 3 were present in this population. No relation was observed between allele 3 and plasma free IGF-I levels. However, plasma total IGF-I levels were 32.1 ng/mL (=4.2 nmol/L) lower in subjects without allele 3 compared to subjects with a copy of this allele (P=0.04, 95% CI –63.4 to –1.8 ng/mL). After adjustment for the effect of allele 3 on plasma thyroid hormone levels, its effect on total IGF-I levels remained significant.

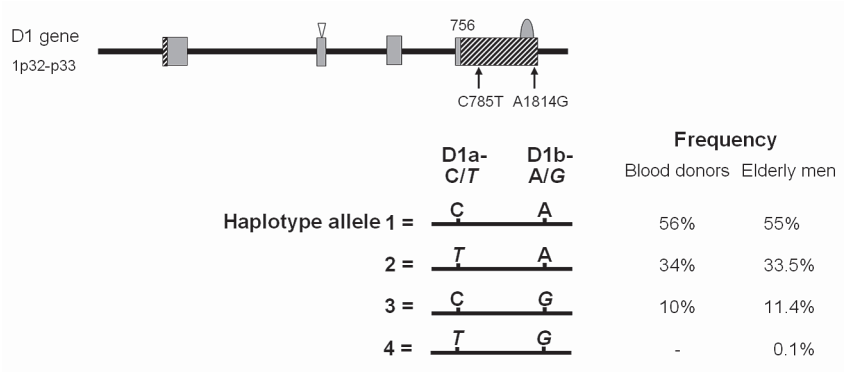



**Figure 1.** Location of the two different polymorphisms in the D1 gene, and the four haplotype alleles that were constructed from these polymorphisms. Frequencies of the haplotype alleles in the two different populations are shown. Size of the different exons is indicated by their scale. The coding sequence is represented by □, whereas ▨ indicates the untranslated region. The UGA codon, representing a selenocysteine, is depicted by ▽. Finally, the SECIS element is indicated by ◠.

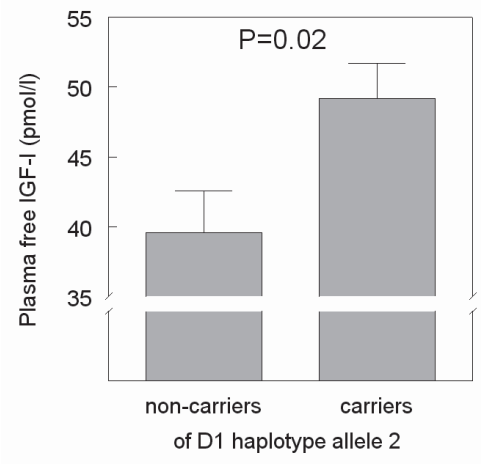



**Figure 2.** Differences in plasma free IGF-I levels between non-carriers and carriers (n=90) of the D1 haplotype 2 allele in a population of 156 blood donors. To convert to pg/ml, multiply with 7.649.

## *Healthy elderly men*

The T variant of D1a had a frequency of 33.6% in this population, whereas the G variant of D1b had a frequency of 11.5%. Again, haplotype alleles were estimated using the computer program 3LOCUS.pas and all subjects were genotyped based on their different haplotype alleles (26, 37). In this population, only one subject with haplotype allele 4 (Fig. 1) was present. In line with a decreased D1 activity

in subjects with haplotype allele 2, a negative effect of this allele was observed on serum T3 levels, independent of age, with evidence for linearity ($P_{trend}$=0.01) (Fig. 3). No effect of this haplotype allele was observed on other serum thyroid hormone parameters or their ratios.

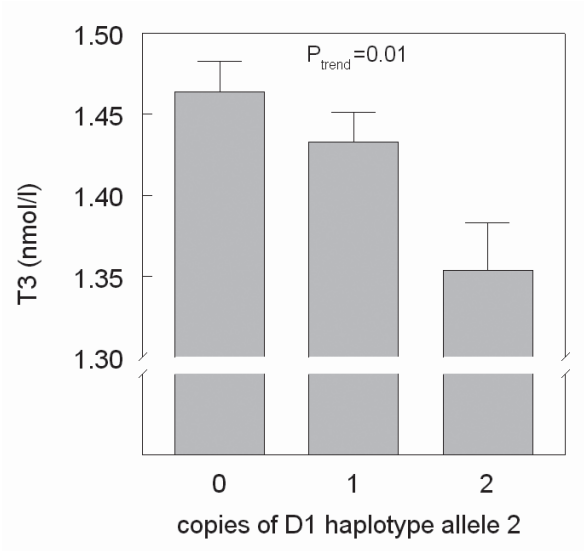



**Figure 3.** Differences in serum T3 levels between subjects with 0, 1, or 2 copies of the of the D1 haplotype 2 allele in a population of 350 elderly men. To convert the values from pmol/L to pg/dl, multiply by 0.0651.

The haplotype allele 2 showed a significant, positive allele dose response with free IGF-I levels ($P_{trend}$=0.01) (Fig. 4a), but not with total IGF-I. Haplotype allele 3 showed an opposite an opposite trend on serum free IGF-I levels ($P_{trend}$=0.08) (Fig. 4b), but had no effect on total IGF-I or T3 levels.

IGF-I levels measured in plasma are low compared to IGF-I measured in serum (38, 39). Therefore, absolute values between this population and the population of elderly men are different and should not be compared.

After the exclusion of all subjects with non-thyroidal illness (combination of a serum T3 < 88 and rT3 > 20.8 ng/dL, n=53), the association of haplotype allele 2 with free IGF-I and T3 levels remained significant (P=0.048 and P=0.003 respectively).

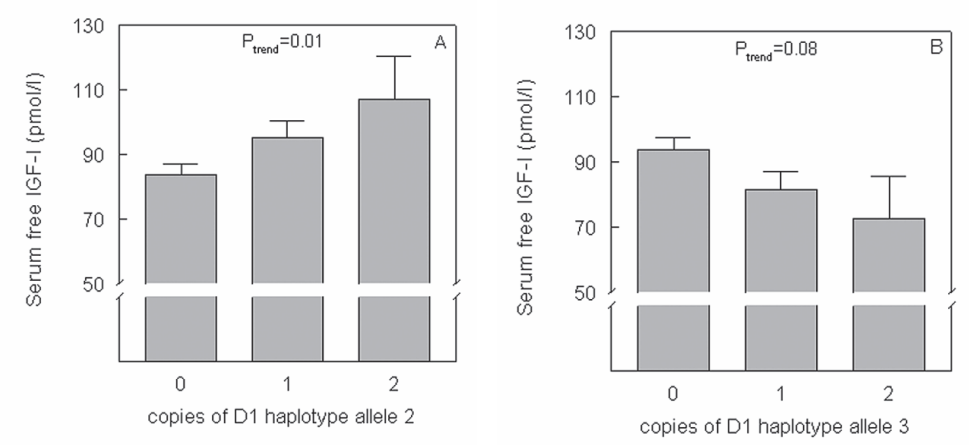



**Figure 4.** Differences in serum free IGF-I levels between subjects with 0, 1, or 2 copies of the of the D1 haplotype 2 allele (A) or the D1 haplotype 3 allele in a population of 350 elderly men. To convert to pg/ml, mutliply with 7.649.

## *Association of SNPs in D1 with physical characteristics*

### *Healthy elderly men*

Carriers of the haplotype allele 2 had a higher lean body mass (50.9 ± 0.4 kg vs. 52.1 ± 0.4 kg, P=0.03, adjusted for age) (Table 3). BMI and fat mass were not different between carriers and non-carriers of haplotype allele 2 (Table 3). Haplotype allele 3 showed no effect on lean body mass. Lean body mass showed a significant negative relation with serum T3 levels (R=-0.16, P<0.01), but not with total or free IGF-I levels.

Muscle strength was also different between carriers and non-carriers of haplotype allele 2. Subjects with one or two copies of allele 2 had a higher isometric grip strength (33.6 ± 0.5 kp vs. 35.0 ± 0.5 kp, P=0.047) and a higher maximum leg extensor strength (100.8 ± 1.6 Nm vs. 104.7 ± 1.5, P=0.07, adjusted for age). Haplotype allele 3 did not show any association with muscle strength in this group of elderly men.

**Table 3.** Physical characteristics of haplotype allele 2 noncarriers and carriers

| | Noncarriers | Carriers | *P* |
|---|---|---|---|
| N | 159 | 191 | |
| Height (cm) | 172.4 | 172.8 | 0.61 |
| BMI (kg/m$^2$) | 25.1 ± 0.2 | 25.5 ± 0.2 | 0.19 |
| WHR | 0.98 ± 0.4[a] | 0.98 ± 0.4 | 0.69 |
| Total Fat Mass (kg) | 20.8 ± 0.4 | 21.0 ± 0.4 | 0.72 |
| Lean Body Mass (kg) | 50.9 ± 0.4 | 52.1 ± 0.4 | 0.03 |
| IGS (kp) | 33.6 ± 0.5 | 35.0 ± 0.5 | 0.05 |
| Maximum LES (Nm) | 100.8 ± 1.6 | 104.7 ± 1.5 | 0.07 |

WHR, Waist-to-hip ratio.
[a]N=158

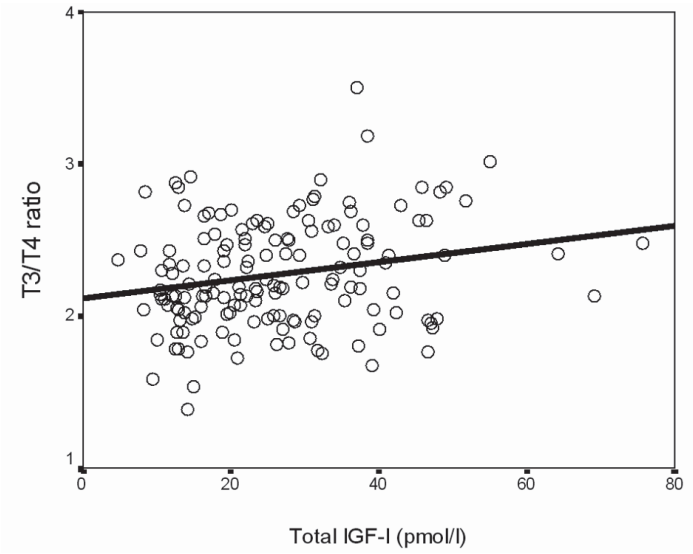



**Figure 5.** Correlation of circulating total IGF-I levels with the T3/T4 ratio in 156 healthy blood donors. To convert the IGF-I values from nmol/L to ng/ml, multiply by 7.6490.

## *Correlation between thyroid hormone parameters and plasma or serum IGF-I levels*

### *Healthy blood donors*

Total and free plasma IGF-I levels were adjusted for age. T4 and FT4 both showed a significant, negative correlation with total IGF-I levels (R=-0.81, P=0.025, and R=-0.25, P=0.002, respectively), whereas the T3/T4 and T3/rT3 ratios showed a

significant, positive correlation with plasma total IGF-I levels (R=0.302, P<0.001, and R=0.180, P=0.026, respectively) (Fig. 5). T3, rT3, TSH, and rT3/T4 ratio did not show any significant correlation with total IGF-I levels. Separate analysis of men and women produced similar results (data not shown).

Free IGF-I showed a negative correlation with T4 (R=-0.26, P=0.001), and TBG (R=-0.31, P<0.001), and a positive correlation with T3/T4 ratio (R=0.21, P0.01). Adjusted for TBG, no correlation between thyroid hormone levels and free IGF-I was observed.

### *Healthy elderly men*

Even in this selected, elderly population, total IGF-I levels were significantly correlated with age (R=-0.104, P=0.037). Adjusted for age, serum T3 was positively correlated with total serum IGF-I levels (R=0.13, P=0.013), whereas TSH, T4, FT4, rT3, and their ratios did not show a significant correlation with total serum IGF-I levels.

Serum free IGF-I was not correlated with age or serum TBG. Free IGF-I levels were not correlated with serum thyroid parameters or their ratios.

## Discussion

We previously demonstrated that two SNPs in D1 are associated with differences in rT3 concentrations in healthy blood donors, but not with serum T3 levels (26). These SNPs are in linkage disequilibrium and haplotype analysis suggested that the haplotype 2 allele results in decreased activity of D1, whereas the haplotype 3 allele results in an increased activity of D1 (26). Because both SNPs are located in the 3'-UTR of the mRNA, a change in the stability of the mRNA is an attractive explanation of their effect. An alternative explanation may be an altered folding of the mRNA, which is necessary for the incorporation of Selenocysteine in the catalytic center of the protein (6). Alternatively, the SNPs could be in linkage disequilibrium with other SNPs located in the coding sequence or in regulatory areas of the gene. In both populations investigated, a significant association of the haplotype allele 2 was observed with free IGF-I levels. Carriers of the haplotype allele 2, with a supposedly lower activity of D1, have higher levels of free IGF-I, whereas no effect of this allele is observed on total IGF-I levels. Since IGF-I has a stimulatory effect on D1 (5), these high levels of free IGF-I could be seen as an adaptation to normalize D1 activity. On the other hand, thyroid hormone has been reported to stimulate IGFBP-1 expression in human hepatoma cells in vitro (40). Although IGFBP-1 is far

less abundant than IGFBP-2 and IGFBP-3, it can account for the greatest changes in free IGF-I levels, since it is usually unsaturated and can vary widely compared to IGFBP-2 and IGFBP-3 (41). IGFBP-1 is mainly produced in the liver (42), and a lower activation of thyroid hormone by liver D1, could result in a lower level of IGFBP-1 and thus a higher level of free IGF-I. No significant effect of haplotype 2 on circulating IGFBP-1 was observed in these subjects (data not shown), but this does not exclude an effect on intracellular hepatic IGF-I.

Free IGF-I may be the major biologically active form of IGF-I (1), and the effect of the D1 haplotype 2 allele on free IGF-I levels was further supported by its effects on several IGF-I related endpoints. Carriers of this D1 haplotype allele had a significantly higher lean body mass, isometric grip strength and a borderline significantly higher maximum leg extensor strength. This D1 genotype effect seems to be mediated by an effect on skeletal muscle, since fat mass and BMI were not different among carriers and non-carriers of the D1 haplotype allele 2. Part of this effect could also be mediated by the effect of haplotype allele 2 on thyroid hormone metabolism, since T4 and T3 showed a negative correlation with lean body mass in this population (43). Both lean body mass and muscle strength are known to be influenced by physical activity. Based on the analysis of two questionnaires and on a test for physical performance, we have no reason to believe that physical activity was different between the groups (data not shown).

GH replacement studies in GH deficient adults suggest that GH stimulates D1 (2-5), and that part of this stimulatory effect could be mediated by IGF-I (5). Liver D1 plays a key-role in the production of plasma T3 from T4 and in the breakdown of the metabolite rT3, but D2 in skeletal muscle may also contribute to serum T3 production (6). The negative correlations we observed in healthy blood donors between IGF-I and T4 and FT4, and the positive correlation of IGF-I with T3/T4 and T3/rT3 are completely in line with a stimulatory effect of IGF-I on liver D1. In elderly men we observed a significant, positive correlation of IGF-I with serum T3, which is also in line with a stimulatory effect of IGF-I on D1, but different from the correlations observed in healthy blood donors. This might be explained by differences in the relative contribution of liver D1 and skeletal muscle D2 to serum T3 production in these different populations (see below). A negative correlation was observed between levels of free IGF-I and TBG in blood donors. This may represent a general biological effect of IGF-I on hormone binding proteins, as IGF-I has been shown to inhibit corticosteroid-binding globulin and sex hormone-binding globulin expression in HepG2 cells (44). However, free IGF-I was not correlated with TBG in elderly men.

Several changes in thyroid hormone concentrations occur during aging: TSH, T3, and FT3 levels show an age-dependent decline, T4 and FT4 levels remain unchanged, whereas rT3 levels increase with age (36). No relation of the polymorphisms in D1 with circulating rT3 levels was observed in the elderly population. In a recent study in critically ill patients, increased levels of rT3 were accompanied by an increased D3 activity (45), and perhaps variations in rT3 production by D3 in elderly subjects masks the effects of D1 on rT3 levels. However, D1 haplotype allele 2 showed an allele dose effect on serum T3 concentrations in elderly men, resulting in lower levels of T3 in carriers of the D1 haplotype 2 allele. This relation remained the same if all subjects with non-thyroidal illness were excluded from the analysis. Haplotype allele 3 showed an opposite relation, which failed to reach significance. Since D1 produces serum T3, this is in line with our hypothesis of a decreased D1 activity in carriers of the haplotype 2 allele, but different from what would be expected based on animal studies. Studies on C3H mice show that these mice, which only have 10-20% D1 activity, have normal levels of circulating T3 that can partially explained by increased levels of plasma T4 (46). Moreover, the contribution of D1 to serum T3 may be even lower in humans than in rodents, which lack D2 in skeletal muscle (6). The different associations found between the two populations, may be explained by the difference in age between these populations (means: 46 vs. 77 years). In young subjects, a decreased T3 production by D1 may be masked by the production of T3 by skeletal muscle D2. Throughout adult life, skeletal muscle size and strength gradually decline (47), resulting in a decrease in D2 expressing skeletal muscle, which is believed to contribute to serum T3 production (6). Furthermore, rT3 levels increase with age and degradation of the D2 protein is accelerated when it is exposed to its own substrates T4 and rT3 (48). As serum rT3 levels are much lower than serum T4 levels, the relative contribution of rT3 to this substrate induced degradation should be very low compared to T4. Although D1 activity also decreases during aging (49, 50), the relative contribution of D2 to serum T3 production may be less important in elderly than in young subjects, resulting in a relatively greater contribution of D1 to serum T3 production at advanced ages.

In conclusion, we show the association of polymorphisms in D1, that result in a decreased D1 activity, with higher levels of free IGF-I in two different populations. These data are supported by the observation that carriers of this polymorphism have a higher lean body mass and muscle strength, with a similar fat mass and BMI. Furthermore, we report that this polymorphism in D1 is associated with serum T3 concentrations in elderly men. This association is in agreement with our previous data in younger blood donors, but might suggest a more important role for D1 in serum T3 production in the elderly.

## Acknowledgements

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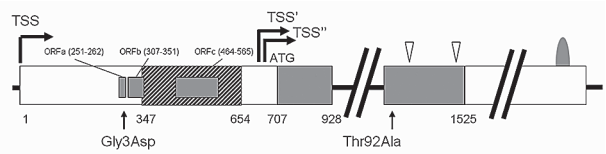


# *Chapter A3*

## A new polymorphism in the type 2 deiodinase (D2) gene is associated with circulating thyroid hormone parameters

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## Abstract

**Introduction:** D2 is important in the regulation of local thyroid hormone bioactivity in certain tissues. D2 in skeletal muscle may also play a role in serum T3 production. In this study we identified a polymorphism in the 5'-UTR of the D2 gene (D2-ORFa-Gly3Asp). We investigated the association of D2-ORFa-Gly3Asp, and of the previously identified D2-Thr92Ala polymorphism, with serum iodothyronine levels.
**Methods:** D2-ORFa-Gly3Asp was identified by sequencing the 5'-UTR of 15 randomly selected individuals. Genotypes for D2-ORFa-Gly3Asp were determined in 156 healthy blood donors (age 46.3±12.2) and 350 ambulant elderly men (age 77.7±3.5) and related to serum iodothyronine and TSH levels.
**Results:** D2-ORFa-$Asp^3$ had an allele frequency of 33.9% in blood bank donors and was associated with serum T4 (Gly/Gly vs Gly/Asp vs Asp/Asp = 7.06±0.14 vs 6.74±0.15 vs 6.29±0.27 μg/dl, P=0.01), FT4 (1.22±0.02 vs 1.16±0.02 vs 1.06±0.04 ng/dl, P=0.001), rT3 (P=0.01) and T3/T4 (P=0.002) in a dose-dependent manner, but not with serum T3 (P=0.59). In elderly men, D2-ORFa-$Asp^3$ had a similar frequency but was not associated with serum iodothyronine levels.
**Conclusion:** This new polymorphism in the 5'-UTR of D2 is associated with iodothyronine levels in blood donors, but not in elderly men. We hypothesize that this might be explained by the decline in skeletal muscle size during aging, resulting in a relative decrease in the contribution of D2 to serum T3 production.


## Introduction

Thyroid hormone is essential for growth, development, and for the regulation of energy metabolism (1). It stimulates metabolic rate by increasing ATP turnover, and by regulating the expression of uncoupling proteins in the mitochondria of fat and skeletal muscle (2, 3). Production of thyroid hormone is regulated by the classic hypothalamic-pituitary-thyroid axis, whereas the biological activity of thyroid hormone, i.e. the availability of T3, is mainly regulated by the three different selenodeiodinases (D1-D3) (4, 5). Tissue distribution and enzymatic activity of the three deiodinases are highly specific, and they all play a different physiological role. D2 is present in brain, pituitary, thyroid, brown adipose tissue, skeletal muscle, and aortic smooth muscle cells in humans (4-10). It converts T4 to T3 by outer ring deiodination, and it is important in the regulation of local thyroid hormone bioactivity in D2-expressing tissues. D2 in skeletal muscle may also contribute to serum T3 production (4, 8).

The mRNA of the D2 gene is unusually long (6-7 kb) compared to the other two selenodeiodinases, containing long 5' and 3' untranslated regions (UTRs) (11-14). The 5'-UTR of the D2 mRNA is ~700 nt long and has an inhibitory effect on D2 translation. It causes a 5-fold reduction in D2 activity in HEK-293 cells (15). This 5'-UTR contains 3 short open reading frames (sORFs) (12-14) (Fig. 1). The most upstream sORF (ORFa) is considered to be primarily responsible for the inhibitory effect of this 5'-UTR, since mutation of the start codon of ORFa completely abolished this inhibitory effect (15).

In this study we scanned the 5'-UTR of D2 for the occurrence of polymorphisms and identified a single nucleotide polymorphism (SNP) in ORFa, the sORF primarily responsible for the inhibitory effect of the 5'-UTR. The association of this SNP, and of the previously identified D2-Thr92Ala (16, 17), with serum iodothyronine levels and their ratios was investigated in a population of blood donors and in a population of elderly men.

## Materials and Methods

### *Study populations*

#### *Healthy blood donors*

Blood was collected from 158 healthy anonymized blood donors at the Sanquin Blood Bank South West Region (Rotterdam, The Netherlands) (17). Informed consent was given by all donors. One subject was excluded because of serum FT4 and TSH levels indicating hyperthyroidism, another because of hypothyroid FT4 and TSH levels. Gender was not documented for one subject. The mean age of the study population was 46.2 ± 12.2 yrs. (mean±SD); 47.4 ± 10.9 yrs. in the males (N=100), and 44.6 ± 13.9 yrs. in the females (N=55). No information on possible thyroid hormone treatment was available. Descriptive statistics of this population are shown in Table 1. No anthropometric data were available of this population. DNA was extracted from 2 ml of blood using the PUREGENE™ DNA Isolation Kit (Gentra Systems, Minneapolis, MN) with slight modifications of the provided protocol. After isolation, DNA concentration was measured at 260 nm, and all samples were diluted to a concentration of 50 ng/µl (stock) and 10 ng/µl (work solution). Purity was determined by measuring the 260/280 nm ratio.

**Table 1.** Descriptive statistics of the population of blood donors

| | Mean ± SD[a] | Minimum | Maximum | Reference values[b] |
|---|---|---|---|---|
| N | 155 | | | |
| Age (years) | 46 ± 12[c] | 19 | 69 | |
| TSH (mU/L) | 1.32 ± 0.75[c] | 0.02 | 5.46 | 0.4 - 4.3 |
| T4 (nmol/L) | 88.0 ± 15.9 | 57.5 | 159.0 | 58 - 128 |
| FT4 (pmol/L) | 15.1 ± 2.4 | 10.4 | 23.4 | 11 - 25 |
| T3 (nmol/L) | 2.0 ± 0.2 | 1.5 | 2.9 | 1.43 - 2.51 |
| RT3 (nmol/L) | 0.31 ± 0.08 | 0.14 | 0.53 | 0.14 - 0.34 |

[a] TSH and iodothyronines were determined in EDTA plasma

[b] Reference values used in our lab, with TSH and iodothyronine levels determined in serum

[c] N=156

### *Healthy elderly men*

DNA was available of 349 men of a cross-sectional, single-center study in 403 independently living men, who were 70 years of age and higher (18). In this study, names and addresses of all male inhabitants of 70 years and older were drawn from the municipal register of Zoetermeer, a medium sized town in the western part of The Netherlands. A total of 1567 men were invited, and after exclusion of subjects who did not live independently and subjects who were not physically or mentally able to visit the study center independently, eventually 403 men participated (25.7%). Participants signed an informed consent. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam. See reference (18) for a more detailed description of this population. 26 subjects were not of Caucasian origin, the majority of them being from Asian origin. See Table 2 for the descriptives of this population. Out of the 349 subjects, 5 received thyroid hormone replacement therapy.

**Table 2.** Descriptive statistics of the population of elderly men

| | N | Mean ± SD | Minimum | Maximum |
|---|---|---|---|---|
| Age (years) | 349 | 77.7 ± 3.5 | 73 | 94 |
| Heigth (cm) | 349 | 172.6 ± 6.5 | 155 | 189 |
| BMI (kg/m$^2$) | 349 | 25.3 ± 3.0 | 15.9 | 34.9 |
| TSH (mU/L) | 334 | 1.14 ± 0.92 | 0.09 | 9.43 |
| T4 (nmol/L) | 333 | 81.0 ± 15.0 | 47.0 | 129.0 |
| FT4 (pmol/L) | 334 | 16.6 ± 3.1 | 8.3 | 31.5 |
| T3 (nmol/L) | 334 | 1.4 ± 0.2 | 0.8 | 2.1 |
| RT3 (nmol/L) | 334 | 0.33 ± 0.09 | 0.15 | 0.74 |

NB All hormone measurements were performed in serum

## *Serum and plasma analyses*

### *Healthy blood donors*

In this population, all hormone measurements were performed in EDTA plasma. T4, FT4, T3 and TSH were measured by chemoluminescence assays (Vitros ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK). rT3 was measured by radioimmunoassay as previously described (19). Intra- and interassay variability coeficients of all the assays were below 11%.

Serum total cholesterol was determined enzymatically on a Hitachi 911 analyzer (Roche Diagnostics, Indianapolis, IN, USA). Both HDL cholesterol (HDL-C) and LDL cholesterol (LDL-C) were determined by a homogeneous assay (Roche Diagnostics) on the Hitachi 911 analyzer (20, 21).

### *Healthy elderly men*

Blood samples were collected in the morning after an overnight fast. Serum was separated by centrifugation and stored at –40 C. TSH was measured using an immunometric technique (Amerlite TSH-30, Ortho-Clinical Diagnostics). Serum T4, T3, and rT3 were all measured by RIA, FT4 by Amerlite MAB FT4 assay (Ortho-Clinical Diagnostics). Intra- and interassay variability coeficients of all the assays were below 11%.

**Table 3.** Conditions and primers used for PCR amplification and sequencing and SBE analysis of D2

| | | PCR primers | Tm | Sequencing and SBE primers |
|---|---|---|---|---|
| | | ***Sequencing of 5'-UTR*** | | |
| | Fw | 5'-TCGCCTAGCTCCTTCCCTGTC-3' | 60C | 5'-ACCTGTATTCAAGTTTCTGCA-3' |
| | Rv | 5'-TAATACCCTTGACAGCCTTCAG-3' | | 5'-AGCCTTCAGGAAATGCTTCCA-3' |
| | Fw | 5'-TCTACCTGCCTTCCAGGATCC-3' | 60C | 5'-GAGAGAGTGTTGGCCATATCAATC-3' |
| | Rv | 5'-CCAGTCTCACTCTCTCCCTTG-3' | | 5'-CCAGGAGCGACTGACTCCTC-3' |
| | Fw | 5'-CGTCAGGGAGACTCACTG-3' | 50C | 5'-GCAGAGAGAGGCACTTTGCAC-3' |
| | Rv | 5'-TTTCAGGATTTAAGATGTTA-3' | | 5'-AGCTGGCGTACTCGTCCCTAA-3' |
| | Fw | 5'-AGAGACTGGACTTAGTCTTGC-3' | 55C | 5'-AGAGGAGACAACTTGGGCTTC-3' |
| | Rv | 5'-TGTCGGCATCGAGGAGGAAGC-3' | | 5'-CCAGACGCAGCGCAGTCCCTC-3' |
| | | ***SBE analysis*** | | |
| D2-Thr92Ala | Fw | 5'-CAGCTATCTTCTCCTGGATACCA-3' | 58C | 5'-(T16)GTGTGGTGCATGTCTCCAGT-3' |
| | Rv | 5'-CTGGTAGGGGAAGTGATCTCG-3' | | |
| D2-ORFa-G/A | Fw | 5'-CGTCAGGGAGACTCACTG-3' | 50C | 5'-($T_{40}$)TGAGCATAGAGACAATGAAAG-3' |
| | Rv | 5'-AGCTGGCGTACTCGTCCCTAA-3' | | |

### *Sequence analysis*

The 5'-UTR of the D2 gene was analyzed for the occurrence of SNPs by sequence analysis in 15 randomly selected blood donors. First, genomic DNA was amplified by polymerase chain reaction (PCR) using the primers listed in Table 3. All primers used in this study were ordered from Invitrogen (Breda, The Netherlands). The PCR conditions were as follows: 5 min at 96 C; 38 cycles of 1 min at 94 C, 1 min at annealing temperature (Table 3), and 1 min at 72 C; and finally 7 min at 72 C. PCR products were verified by agarose gel electrophoresis. Subsequently, they were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Almere, The Netherlands). To increase specificity, sequencing of the PCR products was performed using internal primers (Table 3). Samples were purified using Micro Bio-Spin P-30 Tris columns (Bio-Rad, Veenendaal, The Netherlands), and sequenced directly on an automated ABI 3100 capillary sequencer (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands), using the Big Dye Terminator Cycle Sequencing method (Applied Biosystems).

### *Genotyping*

The genotypes of both populations were determined by Single Base Extension (SBE) analysis as described previously (17). PCR products were generated using the primers listed in Table 2b. Twenty ng of genomic DNA was amplified in a PCR reaction with a total volume of 10 µL. The PCR mixture contained 1x PCR buffer (Invitrogen), 0.2 mM of each dNTP, 1.5 mM MgCl, 2 pmol of each primer, and 0.5 U Taq polymerase (Invitrogen). Annealing temperatures of the PCRs are listed in Table 3. The SBE reactions were performed using the ABI Prism® SNaPshot™ ddNTP Primer Extension Kit (Applied Biosystems) with slight modifications of the protocol provided by the manufacturer. Forward primers were used for SBE analysis of both the D2-Thr92Ala and D2-ORFa-Gly3Asp polymorphisms.

### *Statistical analysis*

Data were analysed using SPSS 10.0.7 for Windows (SPSS Inc., Chicago, IL). Differences between the genotype groups were adjusted for age and/or sex if appropriate and tested by analysis of covariance (ANCOVA) using the general linear model procedure. Bonferroni correction for multiple testing was used where necessary. In case of an allele dose effect we performed a (multiple) lineair regression analysis to quantify the association. To minimize the influence of outliers, logarithmic transformations were performed if appropriate. The effects of the polymorphisms on se-

rum indices of thyroid function in the elderly were analyzed after the exclusion of the subjects on thyroid hormone treatment (n=5). Deviation from Hardy-Weinberg equilibrium was analyzed using a Chi-square test. P values are two-sided throughout, and P=<0.05 was considered significant. Haplotype allele frequencies were estimated using the PHASE program (22).

## Results

### *Identification of new SNPs*

Sequencing of the 5'-UTR of D2 revealed one SNP (GGC to GAC) located in the previously described ORFa (ATG AAA GGC TAA) (Fig. 1) (12). This variation changes the third codon for Gly into a codon for Asp. SBE analysis showed a frequency of 33.9 % of the $Asp^3$ allele in the population of blood donors and of 33.5% in the population of elderly men. Furthermore, the allele frequency of the $Asp^3$ allele of D2-ORFa was not different between the highest and the lowest age quartile in both populations, suggesting that the genotype frequency was independent of age.

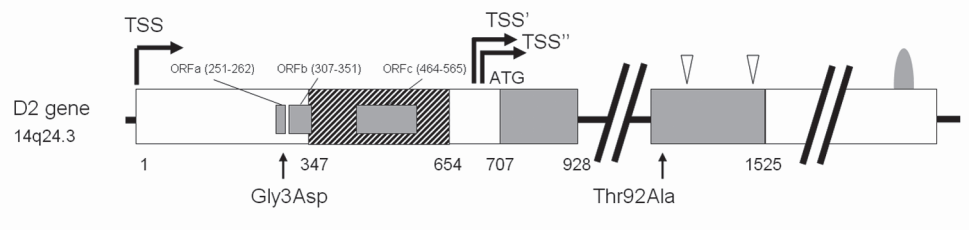


**Figure 1.** Location of the D2-ORFa-Gly3Asp polymorphism in the D2 gene. The coding sequences are represented by ▭, the untranslated region by ▭, whereas ▨ represents an intron that can be spliced out alternatively. TSS is the transcription start site, TSS' and TSS" are two alternative transcription start sites. The two UGA codons, coding for selenocysteine, are depicted by ▽. Finally, ▭ represents the SECIS element.

### *Association of D2-ORFa-Gly3Asp with circulating TSH and iodothyronine levels*

#### *Blood donors*

The $Asp^3$ allele of D2-ORFa was associated with decreasing levels of T4, FT4, and rT3 in a dose-dependent manner: Gly/Gly, 90.82 ± 1.82; Gly/Asp 86.78 ± 1.87; and Asp/Asp 80.96 ± 3.48 nmol/L for T4 (P=0.01, mean ± SE, corresponding to 7.06 ± 0.14, 6.74 ± 0.15, 6.29 ± 0.27 µg/dl, Fig. 2A), Gly/Gly, 15.67 ± 0.28; Gly/Asp 14.86 ± 0.28; and Asp/Asp 13.65 ± 0.53 pmol/L for FT4 (P=0.001, corresponding to 1.22 ± 0.02, 1.16 ± 0.02, 1.06 ± 0.04 ng/dl, Fig 2B), and Gly/Gly, 0.33 ± 0.01; Gly/Asp 0.31

± 0.01; and Asp/Asp 0.28 ± 0.02 nmol/L for rT3 (P=0.01, corresponding to 21 ± 1, 20 ± 1, 18 ± 1 ng/dl, Fig 2D). No effect of the Asp$^3$ allele was observed on plasma T3 levels (P=0.59, Fig 2C) and circulating TSH levels (P=0.54) .

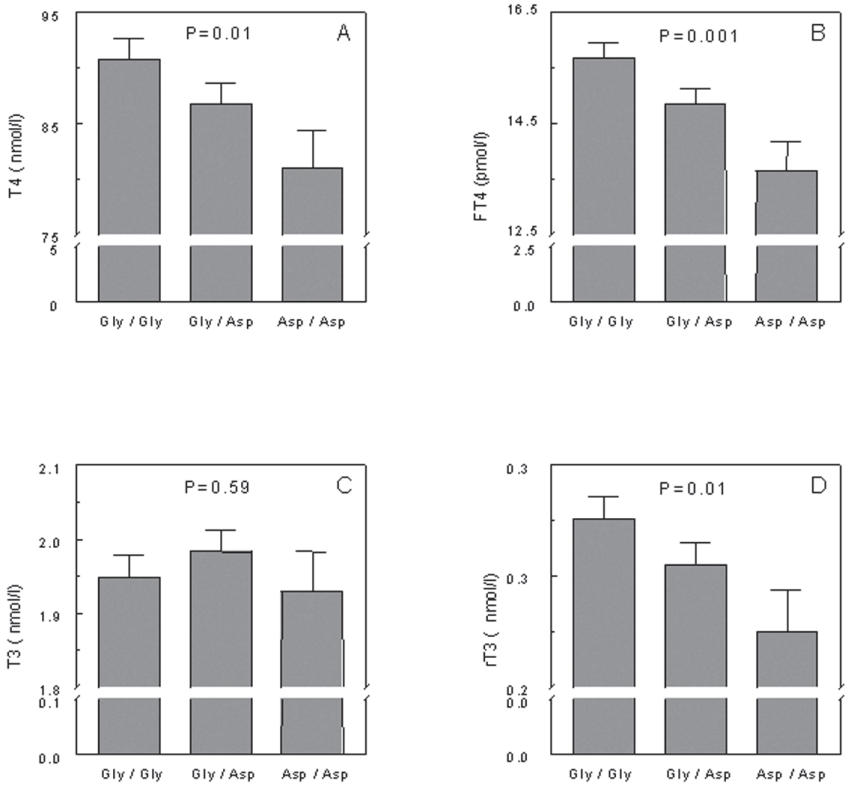


**Figure 2.** Differences in plasma iodothyronine levels between subjects with 0, 1, or 2 copies of the Asp$^3$-allele of D2-ORFa in a population of 156 blood donors. To convert T4 to µg/dl, mutliply by 0.0777, and to convert T3 and rT3 to ng/dl, multiply by 65.1.

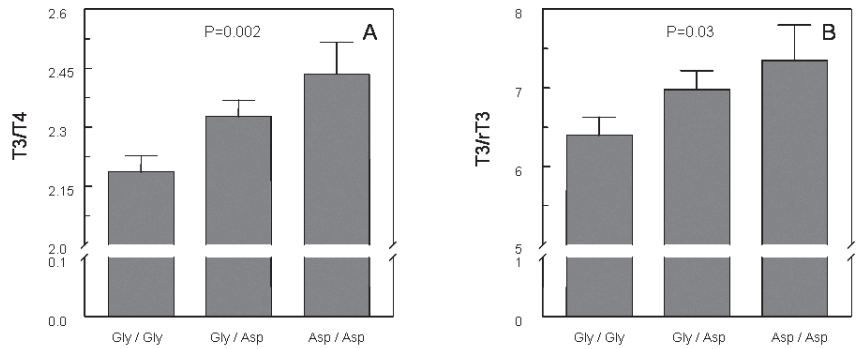


**Figure 3.** Differences in plasma T3/T4 and plasma T3/rT3 ratio between subjects with 0, 1, or 2 copies of the Asp$^3$-allele of D2-ORFa in a population of 156 blood donors.

**Table 4.** Plasma TSH and iodothyronine levels in carriers of the D2-Thr92Ala and in carriers of the D2-ORFa-Gly3Asp variant, in a population of elderly men

| | Thr/Thr | Thr/Ala + Ala/Ala | *P* | Gly/Gly | Gly/Asp + Asp/Asp | *P* |
|---|---|---|---|---|---|---|
| N | 134 | 200 | 0.64# | 148 | 178 | 0.70# |
| TSH (mU/L) | 1.08 ± 0.08 | 1.19 ± 0.07 | 0.99 | 1.20 ± 0.08 | 1.10 ± 0.07 | 0.94 |
| T4 (nmol/L) | 80.42 ± 1.28 | 81.35 ± 1.05[a] | 0.57 | 81.00 ± 1.22[b] | 81.00 ± 1.11 | 0.99 |
| FT4 (pmol/L) | 16.82 ± 0.27 | 16.51 ± 0.22 | 0.38 | 16.67 ± 0.26 | 16.62 ± 0.23 | 0.87 |
| T3 (nmol/L) | 1.42 ± 0.02 | 1.45 ± 0.02 | 0.31 | 1.45 ± 0.02 | 1.43 ± 0.02 | 0.44 |
| RT3 (nmol/L) | 0.34 ± 0.01 | 0.33 ± 0.01 | 0.50 | 0.33 ± 0.01 | 0.33 ± 0.01 | 0.90 |
| T3/T4 | 1.82 ± 0.03 | 1.83 ± 0.03[a] | 0.75 | 1.84 ± 0.03[b] | 1.81 ± 0.03 | 0.51 |
| T3/rT3 | 4.56 ± 0.13 | 4.74 ± 0.11 | 0.28 | 4.71 ± 0.12 | 4.62 ± 0.11 | 0.60 |

Data represent mean ± SE
[a]N=199, [b]N=147
***P*** value for deviation from Hardy Weinberg equilibrium

Furthermore, the $Asp^3$ allele was associated with an increasing T3/T4 ratio (P=0.002) and T3/rT3 ratio (P=0.03), which are both thought to better reflect tissue deiodinase activity (Fig. 3). The difference in T3/T4 ratio corresponds to a 0.36 x SD increase per allele copy.

Separate analysis of male and female subjects gave similar associations of the $Asp^3$ allele with iodothyronine levels in both groups, although it failed to reach significance in the females (n=55).

The D2-Thr92Ala polymorphism was not associated with plasma iodothyronines in this population, as published previously (17).

### *Healthy elderly men*

Due to limited quality and quantity of the DNA, only 341 subjects could be genotyped for both SNPs. In this population, no association of the D2-ORFa-Gly3Asp and/or D2-Thr92Ala variant was observed with serum TSH, iodothyronine levels, or their ratios (Table 4). Association analysis of subjects with 0, 1, or 2 copies of the risk allele gave similar results as analysis of carriers and non-carriers.

## *Association of D2-ORFa-Gly3Asp with plasma cholesterol levels*

### *Blood donors*

The D2-ORFa-related differences in serum thyroid parameters were not accompanied by alterations in cholesterol metabolism, since the D2-ORFa-$Asp^3$ variant was not associated with serum cholesterol, HDL-C or LDL-C in this population (Table 5). The D2-Thr92Ala polymorphism was also not associated with plasma cholesterol levels (data not shown). It should be noted, however, that plasma T4 and T3 were not correlated with plasma cholesterol levels in this population either.

## *Haplotype analysis of D2-Thr92Ala and D2-ORFa-Gly3Asp*

D2-Thr92Ala and D2-ORFa-Gly3Asp were analyzed for linkage disequilibrium in both populations, and haplotype allele frequencies were estimated. Frequency estimates of the 4 different haplotype alleles are shown in Figure 4. The haplotype allele D2-ORFa-$Asp^3$ / $Ala^{92}$ (haplotype allele 4) was only present at very low frequency (4 copies in the blood donors and 8 in the elderly men), suggesting that the D2-ORFa-$Asp^3$ and D2-$Ala^{92}$ allele appear predominantly on different haplotype alleles and are in linkage disequilibrium (P<0.001 for both populations). Because of the low frequency of the haplotype allele 4, the variant alleles are assigned only to frequent

haplotypes (the $Asp^3$ allele to haplotype 2 and the $Ala^{92}$ allele to haplotype 3). Association analysis of haplotype allele 2 with serum thyroid parameters in blood donors gave therefore similar results as the association of the D2-ORFa-$Asp^3$ variant with these parameters.

**Table 5.** Plasma cholesterol levels in carriers and non-carriers of the D2-ORFa-Gly3Asp variant in healthy blood donors

| | Gly/Gly | Gly/Asp + Asp/Asp | *P* |
|---|---|---|---|
| N | 67 | 82 | 0.70# |
| Cholesterol (mmol/L) | 5.65 ± 0.12 | 5.42 ± 0.11 | 0.17 |
| HDL-C (mmol/L) | 1.35 ± 0.04 | 1.30 ± 0.04 | 0.43 |
| LDL-C (mmol/L) | 3.69 ± 0.11 | 3.55 ± 0.10 | 0.34 |
| HDL-C / LDL-C ratio | 0.40 ± 0.02 | 0.41 ± 0.02 | 0.81 |

Data represent mean ± SE, data are adjusted for age and gender
*P* value for deviation from Hardy Weinberg equilibrium

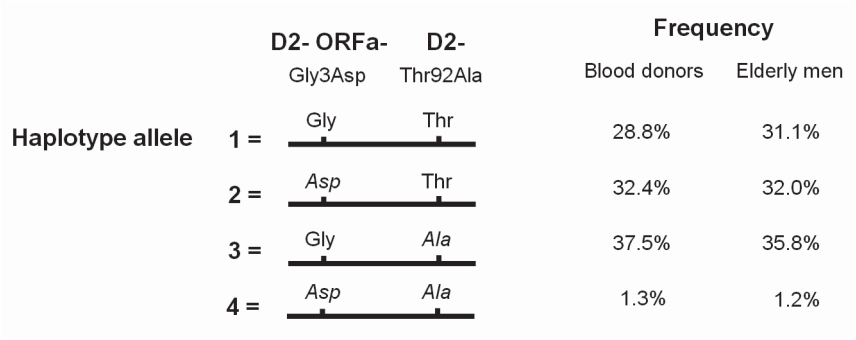


**Figure 4.** The four haplotype alleles that were constructed from the D2-ORFa- Gly3Asp and the D2-Thr92Ala polymorphisms. Frequency estimates of the different haplotype alleles in the two different populations are shown.

## Discussion

In this study we identified a new SNP in the most upstream sORF (ORFa) of the 5'-UTR of D2. ORFa contains only 3 codons and a stop-codon (Met-Lys-Gly) and is primarily responsible for the inhibitory effect of this 5'-UTR on D2 translation (15). The polymorphism identified in this study results in a change of the last codon from Gly to an Asp. In healthy blood donors, this D2-ORFa-$Asp^3$ variant was

associated with lower levels of plasma T4, FT4, and rT3 in a dose-dependent manner, but not with plasma T3 and TSH levels. Pituitary D2 activity is essential for the negative feedback regulation of hypophyseal TSH secretion by circulating T4 (23, 24). D2 knock-out mice have an impaired feedback regulation, resulting in a ~2-fold increased T4 and TSH concentration, but normal circulating T3 (24). The relatively low T4, but unaltered TSH and T3 levels in carriers of the D2-ORFa-Asp$^3$ variant, suggest that the pituitary needs less T4 to produce the same amounts of local T3, and, thus, a normal negative feedback action. These data therefore suggest that the D2-ORFa-Asp$^3$ variant results in a higher D2 activity in the pituitary. As we described previously, the D2-Thr92Ala polymorphism was not associated with plasma iodothyronines in this population (17).

D2 has been identified in skeletal muscle of humans and pigs, especially in the hypothyroid situation (8, 25) (26), although we were not able to measure D2 activity in skeletal muscle of critically ill patients (27). Based on these observations, and on the fact that PTU, an inhibitor of D1 but not of D2, can only partially inhibit T4 to T3 conversion in T4-replaced hypothyroid subjects (28-30), it is believed that D2 is not only involved in the regulation of local thyroid hormone bioactivity, but that it also contributes to circulating iodothyronine levels (4). Plasma iodothyronine levels not only depend on the iodothyronine-metabolizing enzymes, but also, among other things, on thyroid function and plasma-iodothyronine binding capacity. Therefore, the ratios between plasma iodothyronines are thought to better reflect peripheral metabolism of thyroid hormone. The association of the D2-ORFa-Asp$^3$ variant with higher T3/T4 and T3/rT3 ratios supports the hypothesis that D2 contributes to circulating iodothyronine levels. Higher T3/T4 and T3/rT3 ratios in carriers of the D2-ORFa-Asp$^3$ variant suggest that these subjects have a higher D2 activity in tissues such as skeletal muscle, and that this altered D2 activity indeed affects plasma iodothyronine levels. The low serum rT3 levels in carriers of the D2-ORFa-Asp$^3$ variant support this, since D2 can also break down rT3 by outer ring deiodination (4). The D2-ORFa-related differences in plasma thyroid parameters were not accompanied by alterations in cholesterol metabolism, since the D2-ORFa-Asp$^3$ variant was not associated with plasma cholesterol, HDL-C or LDL-C in this population. However, it should be noted that T4 and T3 were not correlated with serum cholesterol levels in this population either.

Only 10% of all mRNA sequences contain a sORF upstream of the major coding sequence (31, 32). Another example of such a gene is the β2 adrenergic receptor, whose 5'-UTR contains a sORF, consisting of 19 codons and a stop-codon, that inhibits the translation of the receptor protein (33). A polymorphism in this sORF,

that alters the last codon from an Arg to a Cys, results in a 2-fold increase in the translational efficiency of the β2 adrenergic receptor (34). Similarly, the D2-ORFa-Gly3Asp polymorphism may very well result in an increased translational efficiency of D2.

There are several potential other mechanisms via which the codon change in ORFa might modulate D2 activity. The polymorphism is located closely to a potential intron, which is spliced out alternatively, producing variant 2 of human D2 mRNA. The D2-ORFa-Gly3Asp polymorphism may thus affect alternative splicing of this intron. Furthermore, because this SNP is located in the 5'-UTR, a change in the stability of the mRNA or in secondary structure may also play a role in the inhibitory effect of 5'-UTR sORFs on translational efficiency (35). Alternatively, the D2-ORFa-Gly3Asp polymorphism could be in linkage disequilibrium with another SNP located in the coding sequence, such as D2-Thr92Ala, or in a regulatory areas of the gene. However, in this study, haplotype analysis indicated that the risk alleles appear largely on separate (frequent) haplotype alleles. The D2-ORFa-Gly3Asp polymorphism does not seem to be related to insulin resistance (unpublished data), in contrast to D2-Thr92Ala, which is associated with insulin resistance (16). Vice versa, D2-Thr92Ala was not related to thyroid hormone levels.

In a population of elderly men, the D2-ORFa-Gly3Asp variant was not associated with plasma iodothyronine levels. The different effects of the D2-ORFa-Asp$^3$ allele in blood donors and elderly men might be explained by the difference in age between the two populations (means: 46 vs. 77 years). Throughout adult life, skeletal muscle size and strength gradually decline, resulting in a decrease in D2 expressing skeletal muscle. Although also D1 activity decreases during aging (36, 37), the relative contribution of D2 to serum iodothyronine production may be less important in elderly than in young subject (38). The association of the D2-ORFa-Gly3Asp variant with plasma iodothyronine levels in blood donors was strongest in the youngest age quartile, whereas this association failed to reach significance in the oldest age quartile, which supports this hypothesis (data not shown).

In conclusion, we identified a polymorphism in ORFa of the 5'-UTR of D2, which is the sORF primarily responsible for the inhibitory effect of the 5'-UTR on D2 expression. This D2-ORFa-Gly3Asp polymorphism is associated with circulating iodothyronine levels in blood donors, but not in elderly men, suggesting an age-dependent effect of this polymorphism.

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# *Chapter A4*

## Additive effects of the Asp727Glu polymorphism in the TSH receptor and the Thr92Ala polymorphism in the type II deiodinase on insulin resistance

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*Submitted*

## Abstract

**Introduction:** It has been shown that a polymorphism in type II deiodinase (D2-Thr92Ala) is associated with insulin resistance. It was also demonstrated that this polymorphism is in weak linkage disequilibrium with a polymorphism in the nearby TSH receptor gene (TSHr-Asp727Glu). Therefore we investigated the association of D2-Thr92Ala and TSHr-Asp727Glu polymorphisms with insulin resistance.
**Methods:** In a population of 320 nondiabetic and 29 diabetic elderly men (age 77.7±3.5 yrs), the associations of D2-Thr92Ala and TSHr-Asp727Glu with serum glucose, insulin, HbA1c, homeostasis model assessment (HOMA), and body composition were investigated.
**Results:** In the non-diabetic subjects, D2-Ala$^{92}$ was associated, in a dose-dependent manner, with higher levels of insulin (P=0.03), glucose (P=0.04), HbA1c (P=0.09), and with a higher HOMA (P=0.03), but not with body composition. Non-diabetic carriers of the TSHr-Glu$^{727}$ allele had a significantly higher glucose (P=0.005), insulin (P<0.001), HbA1c (P=0.003), HOMA (P<0.001), and fat mass (P=0.05). The mean difference in HOMA between carriers and non-carriers of the TSHr-Glu$^{727}$ allele was 47.8%, which is substantially larger than for the D2-Ala$^{92}$ allele (19.2%). The TSHr-Glu$^{727}$ allele, but not the D2-Thr$^{92}$ allele, had a significantly higher frequency in the 29 diabetic subjects than in the remainder of the population (17.2% vs 8.3%, P=0.04).
**Conclusion:** The TSHr-Asp727Glu polymorphism and, to a lesser extent, the D2-Thr92Ala polymorphism were associated with insulin resistance in a population of non-diabetic elderly men with evidence for an additive effect.

## Introduction

Thyroid hormone is essential for the regulation of energy metabolism (1). It stimulates metabolic rate by increasing ATP turnover, and by regulating the expression of uncoupling proteins in the mitochondria of fat and skeletal muscle (2, 3). Production of thyroid hormone is regulated by the classic hypothalamus-pituitary-thyroid axis, in which thyroid stimulating hormone (TSH) is secreted by the pituitary and interacts with the TSH receptor (TSHr) in the thyroid. The biological activity of thyroid hormone, i.e., the availability of T3, is mainly regulated by the three different selenodeiodinases (D1-D3), who all play a different physiological role (4, 5).

In humans, D2 is present in brain, pituitary, thyroid, brown adipose tissue, and skeletal muscle (4-8). It converts T4 to T3 by outer ring deiodination, and it is important in the regulation of local thyroid hormone bioactivity in D2-expressing

tissues (4). In a recent study, it was shown that an amino acid substittion polymorphism in D2 (D2-Thr92Ala) is associated with insulin resistance (9). Carriers of the D2-Ala$^{92}$ variant had a decreased glucose disposal rate and a tendency towards a higher fasting insulin level, but fasting glucose and BMI were not different compared to non-carriers of this variant (9). *In vitro* analysis of the D2-Thr92Ala variant showed no alteration in deiodinase activity however, suggesting that the association with insulin resistance might be explained by linkage to a different polymorphism (10).

The D2 gene and TSHr gene are both located on chromosome 14 separated by a distance of 0.74 megabases (Figure 1). A recent study of 126 Russian families with Graves' disease showed that the D2-Thr92Ala polymorphism is in weak pairwise linkage disequilibrium with a polymorphism in the TSHr (Asp727Glu) (11). In this study, the D2-Ala$^{92}$ / TSHr-Asp$^{727}$ haplotype was preferentially transmitted from parents to affected siblings (11). The TSHr is not only expressed in the thyroid, but also, among other tissues, in adipose tissue (12-16). Furthermore, the TSH receptor protein that is expressed in abdominal preadipocytes has been shown to be functional (14), and TSH is able to induce proliferation and inhibit differentiation of cultured rat preadipocytes (17). We therefore speculated that the association of the D2-Thr92Ala polymorphism with insulin resistance could (partially) be explained by linkage with the TSHr-Asp727Glu polymorphism.

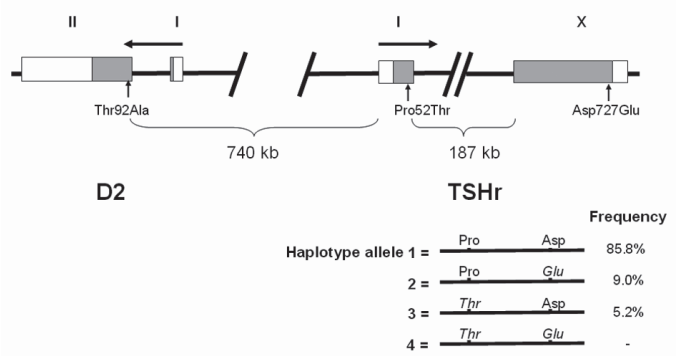



**Figure 1.** Exon I and II of the D2 gene and exon I and X of the TSHr gene on chromosome 14q31. The arrows represent the orientation of the genes. Location of the D2-Thr92Ala, TSHr-Pro52Thr, and TSHr-Asp727Glu polymorphisms is shown. Frequencies of the four haplotype alleles constructed from the TSHr-Pro52Thr and TSHr-Asp727Glu polymorphisms, as observed in the study population are shown below. The coding sequence is represented by ▭, whereas ▭ indicates the untranslated region.

In this study, we analyzed in a population of elderly men if the D2-Thr92Ala and/or the TSHr-Asp727Glu were associated with insulin resistance, and if these associations could be explained by linkage between the two polymorphisms. The previously published TSHr-Pro52Thr was also analyzed.

## Research design and Methods

### *Study population*

DNA was available of 349 men of a cross-sectional, single-center study in 403 independently living men, who were 70 years of age and higher (18). In this study, names and addresses of all male inhabitants of 70 years and older were drawn from the municipal register of Zoetermeer, a medium sized town in the midwestern part of The Netherlands. A total of 1567 men were invited, and after exclusion of subjects who did not live independently and subjects who were not physically or mentally able to visit the study center independently, eventually 403 men participated (25.7%). Participants signed an informed consent. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam. See reference (18) for a more detailed description of this population. 26 subjects were not of Caucasian origin, the majority of them being from Asian origin. Height and weight were measured in standing position without shoes. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters. Lean body mass and fat mass were measured by dual-energy x-ray absorptiometry (19, 20).

See Table 1 for the descriptives of this population. Out of the 349 subjects, 5 received thyroid hormone replacement therapy, and 29 of them had diabetes.

### *Serum analyses*

Blood samples were collected in the morning after an overnight fast. Serum was separated by centrifugation and stored at –40 C. TSH was measured using an immunometric technique (Amerlite TSH-30, Ortho-Clinical Diagnostics). Serum T4, T3, and rT3 were all measured by RIA, FT4 by Amerlite MAB FT4 assay (Ortho-Clinical Diagnostics). Intra- and interassay variability coeficients of all the assays were below 11%. Insulin was measured by a commercially available RIA (Pharmacia, Freyburg, Germany). Insulin sensitivity was further estimated by homeostasis model assessment (HOMA= fasting insulin (mIU/ml) X fasting glucose (mmol/L) / 22.5), as recently described and validated (21, 22).

**Table 1**. Descriptive statistics of the population of elderly men

| | |
|---|---|
| Age (years) | 77.7 ± 3.5 (349) |
| Height (cm) | 172.6 ± 6.5 (349) |
| Weight (kgs) | 75.5 ± 10.3 (349) |
| BMI (kg/m2) | 25.3 ± 3.0 (349) |
| WHR | 0.98 ± 0.05 (346) |
| TSH (mU/L) | 0.96 (0.61 – 1.40) (334) |
| T4 (nmol/L) | 81.0 ± 15.0 (333) |
| FT4 (pmol/L) | 16.6 ± 3.1 (334) |
| T3 (nmol/L) | 1.4 ± 0.2 (334) |
| RT3 (nmol/L) | 0.33 ± 0.09 (334) |
| No. receiving TH [%] | 5 [1.4%] (349) |
| Glucose (mmol/L) | 5.8 ± 2.7 (348) |
| Insulin (IU/L) | 8.9 ± 4.3 (347) |
| HbA1c (%) | 5.8 ± 0.9 (348) |
| HOMA [IQR] | 1.92 [1.40 -2.67] (346) |
| No. with diabetes [%] | 29 [8.3%] (348) |
| Lean Body Mass (kgs) | 51.6 ± 5.7 (348) |
| Fat Mass (kgs) | 20.9 ± 5.5 (349) |

Data are shown as mean ± SD, as median [IQR], or as no. [%].
The number of patients of whom data were available is shown between brackets.
IQR, Interquartile range; TH, thyroid hormone

## *Genotyping*

The genotypes of the population was determined by Single Base Extension (SBE) analysis as described previously (10). PCR products were generated using the primers listed in Table 2. Twenty ng of genomic DNA was amplified in a PCR reaction with a total volume of 10 μL. The PCR mixture contained 1x PCR buffer (Invitrogen), 0.2 mM of each dNTP, 1.5 mM MgCl, 2 pmol of each primer, and 0.5 U Taq polymerase (Invitrogen). Annealing temperatures of the PCRs are listed in Table 2. The SBE reactions were performed using the ABI Prism® SNaPshot™ ddNTP Primer Extension Kit (Applied Biosystems) with slight modifications of the protocol provided by the manufacturer. Forward primers were used for SBE analysis of the D2-Thr92Ala and TSHr-Asp727Glu polymorphisms, the reverse primer was used for the TSHr-Pro52Thr polymorphism.

**Table 2**. Conditions and primers used for PCR amplification and SBE analysis

| | | PCR primers | Tm | SBE primers |
|---|---|---|---|---|
| D2-Thr92Ala | Fw | 5'-CAGCTATCTTCTCCTGGATACCA-3' | 58C | 5'-(T16)GTGTGGTGCATGTCTCCAGT-3' |
| | Rv | 5'-CTGGTAGGGGAAGTGATCTCG-3' | | |
| TSHr-Pro52Thr | Fw | 5'-ATTTCGGAGGATGGAGAAATA-3' | | |
| | Rv | 5'-GCAGATGCCCTTGATCTCTG-3' | 58C | 5'-(T28)CTTCAGAGTCTGCGTACTGG-3' |
| TSHr-Asp727Glu | Fw | 5'-AACGCCAGGCTCAGGCATAC-3' | 60C | 5'-(T34)GTTCAAAAGGTTACCCACGA-3' |
| | Rv | 5'-AAGTTCCCCTACCATTGTGA-3' | | |

### *Statistical analysis*

Data were analysed using SPSS 10.0.7 for Windows (SPSS Inc., Chicago, IL). Differences between the genotype groups were adjusted for age and tested by analysis of covariance (ANCOVA) using the general linear model procedure. 46 subjects in this study were homozygous for D2-Ala$^{92}$ allele, none were homozygous for the TSHr-Thr$^{52}$ allele, and only three were homozygous for the TSHr-Glu$^{727}$ allele. Therefore the SNPs in the TSH receptor were only analyzed as carriers vs non-carriers. In case of an allele dose effect for the D2-Thr92Ala polymorphism, we performed a (multiple) lineair regression analysis to quantify the association. The effects of the polymorphisms on serum indices of thyroid function in the elderly were analyzed after the exclusion of the subjects on thyroid hormone treatment (n=5). Because of the interaction of diabetes and antidiabetic drugs on glucose and insulin metabolism, the effect of the two polymorphisms on relative insulin resistance was analyzed after the exclusion of all subjects with diabetes (n=29). Deviation from Hardy-Weinberg equilibrium was analyzed using a Chi-square test. P values are two-sided throughout, and $P<0.05$ was considered significant. Haplotype allele frequencies were estimated using the PHASE program (version 2) (23).

## Results

### *Association of polymorphisms with serum TSH and iodothyronine levels*

D2-Thr92Ala had an allele frequency of 37.1 %, TSHr-Pro52Thr of 5.2 %, and TSHr-Asp727Glu of 9.0 %. These frequencies are similar to the allele frequencies

we previously found in a younger population of blood donors (10), and all three polymorphisms followed Hardy-Weinberg equilibrium.

Plasma TSH and iodothyronine levels were not different between carriers and non-carriers of the TSHr-Thr$^{52}$ allele (Table 3). Carriers of the TSHr-Glu$^{727}$ allele had a significantly lower serum rT3, whereas there was no significant difference in serum TSH or other iodothyronines (Table 3). As we described previously, the D2-Thr92Ala polymorphism was not associated with serum thyroid parameters in this population either (24).

**Table 3.** Plasma TSH and iodothyronine levels in carriers of the TSHr-Pro52Thr and in carriers of the TSHr-Asp727Glu variant

| | Pro/Pro | Pro/Thr | *P* | Asp/Asp | Asp/Glu + Glu/Glu | *P* |
|---|---|---|---|---|---|---|
| N | 300 | 34 | 0.31# | 276 | 58 | 0.92# |
| TSH (mU/L) | 1.15 ± 0.05 | 1.10 ± 0.16 | 0.91 | 1.12 ± 0.06 | 1.26 ± 0.12 | 0.39 |
| T4 (nmol/L) | 81.2 ± 0.9[a] | 78.8 ± 2.5 | 0.37 | 81.6 ± 0.9[b] | 77.9 ± 1.9 | 0.08 |
| FT4 (pmol/L) | 16.6 ± 0.2 | 16.9 ± 0.5 | 0.64 | 16.7 ± 0.2 | 16.4 ± 0.4 | 0.58 |
| T3 (nmol/L) | 1.45 ± 0.01 | 1.39 ± 0.04 | 0.19 | 1.44 ± 0.01 | 1.42 ± 0.03 | 0.39 |
| rT3 (nmol/L) | 0.33 ± 0.01 | 0.33 ± 0.02 | 0.80 | 0.34 ± 0.01 | 0.31 ± 0.01 | **0.03** |
| T3/T4 | 1.83 ± 0.02[a] | 1.80 ± 0.06 | 0.67 | 1.82 ± 0.02[b] | 1.87 ± 0.05 | 0.33 |
| T3/rT3 | 4.67 ± 0.09 | 4.61 ± 0.26 | 0.83 | 4.61 ± 0.09 | 4.96 ± 0.20 | 0.11 |

*Association of polymorphisms with relative insulin resistance and body composition*

29 patients of the studied population had diabetes. The TSHr-Glu$^{727}$ allele had a twofold higher frequency in the subjects with diabetes than in the 320 subjects without diabetes (17.2 % vs 8.3%, P=0.04). The frequency of the D2-Ala$^{92}$ and of the TSHr-Thr$^{52}$ alleles were not different between subjects with and without diabetes (P=0.35 and P=0.20, respectively). Because of the use of antidiabetic drugs, subjects with diabetes were excluded from all further analyses.

D2-Ala$^{92}$ was, in a dose-dependent manner, associated with increased levels of fasting insulin (8.25 ± 0.36 vs 8.96 ± 0.34 vs 9.80 ± 0.64 IU/L in non-diabetic subjects with 0, 1, or 2 copies of the Ala$^{92}$ allele, P=0.03; 18.8 % difference between the extreme genotypes), and fasting glucose (5.29 ± 0.10 vs 5.57 ± 0.09 vs 5.57 ± 0.17 mmol/L, P=0.04; 5.3%) (Table 4). Hemoglobin A1c (HbA1c) showed an upward trend, but failed to reach significance (P=0.09). Furthermore, D2-Ala$^{92}$ was associated with an increased HOMA in a dose-dependent manner (1.98 ± 0.12 vs

2.29 ± 0.12 vs 2.58 ± 0.22, P=0.03; 30.3 %) (Fig. 2a). The mean difference in HOMA between carriers and non-carriers of the D2-Ala$^{92}$ allele was 19.2%.

The TSHr-Pro52Thr polymorphism was not associated with glucose, insulin, HbA1c, or HOMA (Table 4). Carriers of the TSHr-Glu$^{727}$ allele, however, had a 8.7% higher glucose (P=0.005), a 29.4% higher insulin (P<0.001), a 6.1% higher HbA1c (P=0.003), and a 47.8 % higher HOMA (P<0.001) (Table 4 and Figure 2b). The difference for these characteristics between carriers and non-carriers of this polymorphism was substantially larger than for the D2-Thr92Ala polymorphism, with an increase in HOMA of 0.7 SD in carriers of the TSHr-Glu$^{727}$ allele.

The D2-Thr92Ala and TSHr-Pro52Thr polymorphisms were not associated with lean body mass or fat mass, whereas carriers of the TSHr-Glu$^{727}$ allele had a 5.5% higher fat mass than non-carriers (Table 4).

### *The effect of linkage between D2-Thr92Ala, TSHr-Pro52Thr and TSHr-Asp727Glu on the association with relative insulin resistance*

TSHr-Pro52Thr and TSHr-Asp727Glu were analyzed for linkage disequilibrium, and frequencies of the different haplotype alleles are shown in Figure 1. The haplotype allele TSHr-Thr$^{52}$ / TSHr-Glu$^{727}$ (haplotype allele 4) was not observed in this population. Association analysis of haplotype allele 3, which carries the TSHr-Glu$^{727}$ variant, with serum thyroid parameters or with characteristics of relative insulin resistance gave exactly the same results as the association analyses of the TSHr-Glu$^{727}$ allele with these characteristics (Table 3 and 4). Similarly, there was no difference between the analysis of the TSHr-Thr$^{52}$ allele and the analysis of the haplotype 2 allele.

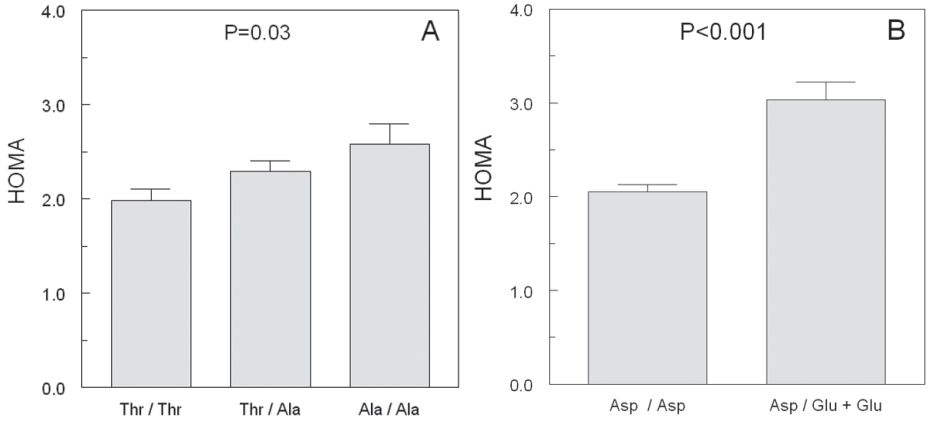
The D2-Thr92Ala and TSHr-Pro52Thr polymorphisms were not associated with lean body mass or fat mass, whereas carriers of the TSHr-Glu$^{727}$ allele had a 5.5% higher fat mass than non-carriers (Table 4).

**Table 4.** The effect of D2-Thr92Ala, TSHr-Pro52Thr, and TSHr-Asp727Glu on relative insulin resistance and anthropometric parameters

| | Thr/Thr | Thr/Ala + Ala/Ala | % difference | *P* | Pro/Pro | Pro/Thr | *P* |
|---|---|---|---|---|---|---|---|
| Glucose (mmol/L) | 5.29 ± 0.10 | 5.57 ± 0.08 | 5.3 % | **0.02** | 5.49 ± 0.07 | 5.19 ± 0.20 | 0.15 |
| Insulin (IU/L) | 8.25 ± 0.37 | 9.14 ± 0.30 | 10.8 % | 0.06 | 8.89 ± 0.24 | 7.78 ± 0.74 | 0.15 |
| HbA1c (%) | 5.55 ± 0.07 | 5.72 ± 0.05 | 3.1 % | **0.05** | 5.66 ± 0.04 | 5.63 ± 0.14 | 0.85 |
| HOMA | 1.98 ± 0.12 | 2.36 ± 0.10 | 19.2 % | **0.03** | 2.25 ± 0.08 | 1.83 ± 0.25 | 0.16 |
| BMI (kg/m2) | 25.2 ± 0.3 | 25.5 ± 0.2 | | 0.41 | 25.4 ± 0.2 | 25.1 ± 0.5 | 0.57 |
| WHR | 0.984 ± 0.004 | 0.979 ± 0.003 | | 0.38 | 0.981± 0.003 | 0.982 ± 0.008 | 0.92 |
| Lean Mass (kgs) | 51.7 ± 0.5 | 51.7 ± 0.4 | | 0.98 | 51.5 ± 0.3 | 53.0 ± 1.0 | 0.17 |
| Fat Mass (kgs) | 20.8 ± 0.5 | 21.3 ± 0.4 | | 0.42 | 21.1 ± 0.3 | 21.3 ± 1.0 | 0.83 |

| | Asp/Asp | Asp/Glu + Glu/Glu | % difference | P |
|---|---|---|---|---|
| Glucose (mmol/L) | 5.38 ± 0.07 | 5.85 ± 0.15 | 8.7 % | **0.005** |
| Insulin (IU/L) | 8.39 ± 0.25 | 10.86 ± 0.57 | 29.4 % | **<0.001** |
| HbA1c (%) | 5.60 ± 0.05 | 5.94 ± 0.10 | 6.1 % | **0.003** |
| HOMA | 2.05 ± 0.08 | 3.03 ± 0.19 | 47.8 % | **<0.001** |
| BMI (kg/m2) | 25.3 ± 0.2 | 25.8 ± 0.4 | | 0.32 |
| WHR | 0.979 ± 0.003 | 0.990 ± 0.007 | | 0.14 |
| Lean Mass (kgs) | 51.5 ± 0.3 | 52.4 ± 0.8 | | 0.29 |
| Fat Mass (kgs) | 20.9 ± 0.3 | 22.5 ± 0.8 | 5.5 % | **0.05** |

Data are shown as mean ± SE.




D2-Thr92Ala TSHr-Asp727Glu

**Figure 2.** Differences in HOMA score between subjects with 0, 1, or 2 copies of the D2-Ala$^{92}$ allele (A) and between carriers and non-carriers of the TSHr-Glu$^{727}$ allele (B) in a population of 349 elderly men, after the exclusion of all subjects with diabetes (n=29).

Linkage analysis for the D2-Thr92Ala and the TSHr-Asp727Glu polymorphisms using the PHASE program showed a D' of ~ 0.50, which is low and similar to a previously published study (D'=0.56) (11). Since haplotype analysis is not meaningful when the D' is this low, we performed a combined analysis of the two polymorphisms to analyze possible additive or multiplicative effects. Both in carriers and in non-carriers of the TSHr-Glu727 allele, the D2-Ala92 allele resulted in higher levels of glucose, insulin, HbA1c and in a higher HOMA, and vice versa (Fig. 3). We analyzed covariance of these parameters with both the D2-Thr92Ala and the TSHr-Asp727Glu polymorphism in a regression model, together with an interaction term (D2-Thr92Ala x TSHr-Asp727Glu). This showed no evidence for a multiplicative interaction between the D2-THr92Ala and the TSHr-Asp727Glu polymorphisms in their effect on insulin resistance (P=0.47, P=0.65, P=0.52, and P=0.26 for the interaction term for glucose, insulin, HbA1c and HOMA, respectively). However, both polymorphisms were independently associated with insulin resistance if the interaction term was excluded from the model, with evidence for an additive effect (See figure 3) (P=0.03 and P=0.007 for the D2-Thr92Ala and the TSHr-Asp727Gly polymorphisms on glucose in this model, respectively, P=0.09 and P<0.001 for insulin, P=0.07 and P=0.004 for HbA1c, and P=0.03 and P<0.001 for HOMA).

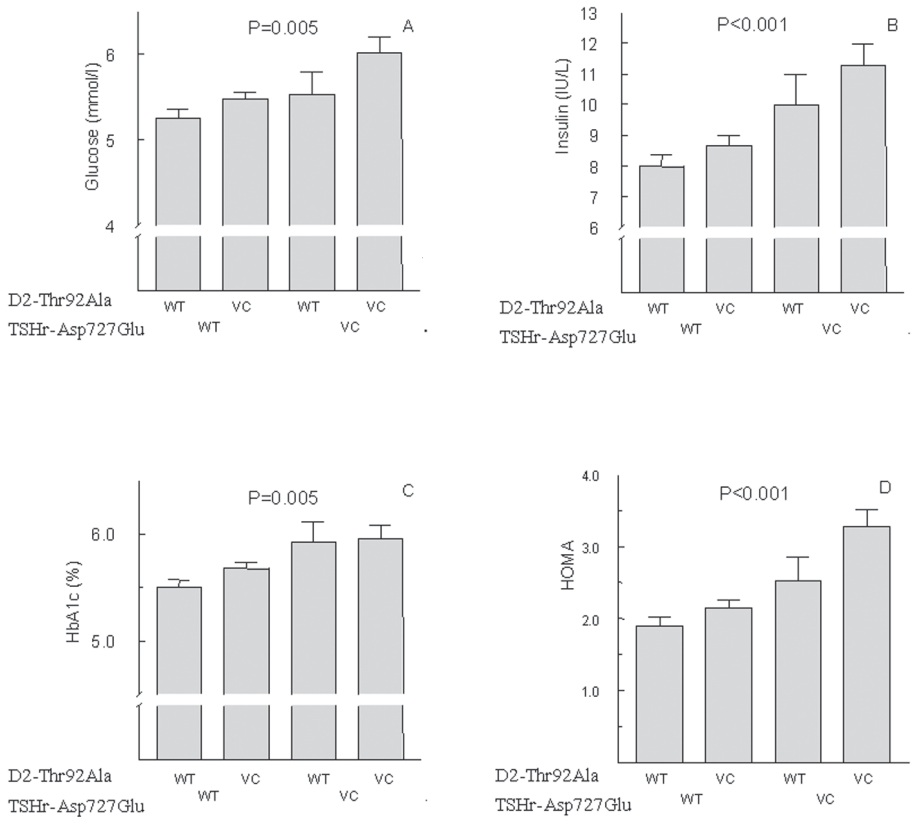



**Figure 3.** Differences in serum glucose (A), insulin (B), HbA1c (C), and HOMA score (D) between non-carriers (WT) and carriers (VC) of the D2-Ala$^{92}$ allele, subdivided in carriers (VC) and non-carriers (WT) of the TSHr-Glu$^{727}$ allele in a population of 349 elderly men, after the exclusion of all subjects with diabetes (n=29). P-values are for trends.

## Discussion

In this population of elderly men, no associations of the D2-Thr92Ala, the TSHr-Pro52Thr, and the TSHr-Asp-727Glu polymorphisms were found with serum TSH, T4, or T3 levels. Nevertheless, both the D2-Ala92 allele and the TSHr-Glu727 allele were associated with different parameters of insulin resistance. The TSHr-Glu727 allele was also associated with fat mass and had a two fold higher frequency in patients who were diagnosed with diabetes than in those who were not. We used fasting glucose, insulin, HbA1c, and the HOMA index as estimates of insulin resistance. The HOMA index shows a good correlation with a euglycemic clamp study

(21, 25), and is therefore considered a good proxy for cohort and epidemiological studies (26).

The association of both the D2-Thr92Ala and the TSHr-Asp727Glu polymorphism with insulin resistance, which was observed in non-diabetic subjects in our study, might be caused by a) an effect of both SNPs independent of each other, b) or by the functional relationship between the TSHr and D2, since stimulation of the TSHr results in an increased activity of D2 in the thyroid, brown adipose tissue (BAT), and osteoblasts (27-29). Based on our HapMap analysis (data not shown) we can exclude linkage disequilibrium to explain the association between the two polymorphisms, because the 700 kb area between the two polymorphisms covers multiple linkage disequilibrium blocks.

Non-diabetic carriers of the D2-Ala92 variant had higher levels of fasting insulin and glucose, higher HOMA values, and a tendency towards a higher HbA1C than non-carriers of this variant. This association is independent of an effect of the polymorphism on serum hormone levels, since the D2-Ala92 allele is not associated with serum thyroid hormone levels (10, 24). The association with relative insulin resistance, that we found, is in agreement with a previous study, in which the D2-Ala92 was associated with a decreased glucose disposal rate in 135 caucasian nondiabetic women (9). Although in vitro analysis of D2-Thr92Ala showed no effect on expression of D2 activity in transfected COS cells (10), this variant has now been found to be associated with relative insulin resistance in two unrelated study populations.

D2 is expressed in skeletal muscle and in brown fat in humans, but is not known to be expressed in white fat (4, 7). Thyroid hormone is important in the augmentation of catecholamine-stimulated lipolysis (30), and an inactivating thyroid hormone receptor alpha mutation results in a phenotype of insulin resistance in mice (31). Furthermore, T3 stimulates the transcription of the muscle/fat-specific insulin-sensitive glucose transporter GLUT4 (32-35). A decreased D2 activity in insulin-sensitive tissues such as adipose tissue and skeletal muscle, resulting in a decreased availability of local T3, may thus explain the association of D2-Thr92Ala with relative insulin resistance (9).

Non-diabetic carriers of the TSHr-Glu727 variant had higher levels of fasting insulin and glucose, a higher HbA1c and HOMA, and also had an increased fat mass. Transfection studies of this variant receptor have resulted in conflicting data regarding the cAMP response to TSH (36, 37). The TSHr-Asp727 variant has been found to be associated with decreased serum TSH levels in blood donors (10), an observation we have recently confirmed in another unrelated study population

(unpublished data). These data support the findings showing an increased cAMP response of the TSHr-Glu727 variant to TSH (36), because less TSH would be required to achieve a normal thyroid hormone production.

The functional TSH receptor protein is not only expressed in the thyroid, but also in human adipose tissue (12-16), and TSH is able to induce proliferation and inhibit differentiation in cultured rat preadipocytes (17). A more active TSH receptor, and similar TSH levels in carriers of the TSHr-Glu727 allele in this population, may thus result in an increased adipogenesis, leading to an increased fat mass and making subjects more vulnerable to the development of insulin resistance. Furthermore, a different ligand for the TSHr named thyrostimulin has recently been identified (38, 39). The variant TSH receptor may also have an increased response to thyrostimulin. The Glu727 variant receptor demonstrated an altered responsiveness to TSH with increased cAMP generation, which indicates an altered biological behavior with respect to adenylyl cyclase activation (36). The effect of this variant may be different in different tissues such as thyroid and fat, dependent on the cell-specific G proteins involved in signal transduction.

It has been described that the D2-Thr92Ala and TSHr-Asp727Glu polymorphism are in weak linkage disequilibrium (11). The D2 gene and TSHr gene are located 0.74 megabases from each other. The genes show an opposite orientation, with their 5'UTRs facing inward. Based on our analysis of HapMap data (not shown) it can be excluded that the observed associations are caused by linkage of the D2-Thr92Ala and the TSHr-Asp727Glu polymorphisms to a third polymorphism. In addition, we did find that the association of D2-Thr92Ala with insulin resistance could not be explained by linkage to the TSHr-Asp727Glu polymorphism, since our regression analyses suggest independent and additive effects. D2-Ala92 carriers had a tendency towards a higher glucose, insulin, HbA1c and HOMA, independent of the TSHr-Asp727Glu polymorphism, and we observed an additive effect. The combined analysis suggested a stronger effect of the TSHr-Glu727 allele than of the D2-Ala92 allele on insulin resistance.

The epidemiological observations seem to be supported by what is known about biological function and interplay of these proteins. D2 and the TSH receptor are both expressed in pituitary, thyroid, BAT and osteoblasts, and there is a close functional relation between the two proteins (4, 7, 27, 29). Stimulation of the TSH receptor results in an increased expression of D2 (4, 27, 29), suggesting that an increased activity of the TSHr will result in an increase in D2 activity. However, arguing against this functional interaction are results suggesting that the D2- Ala92 allele has a lower activity.

In conclusion, the TSHr-Asp727Glu polymorphism, and to a lesser extent the D2-Thr92Ala polymorphism, are associated with insulin resistance in a population of elderly men. Although the two polymorphisms have been described to be in weak linkage disequilibrium, our data suggest an independent association of both polymorphisms with insulin resistance with evidence for an additive effect.

## Acknowledgements

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# *Chapter A5*

## Polymorphisms in the type II deiodinase are associated with atrial fibrillation in elderly subjects

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*Submitted*

## Abstract

**Introduction:** Hyperthyroid patients have tachycardia and often atrial fibrillation (AF). Overexpression of D2 in the mouse heart causes cardiac-specific thyrotoxicosis, and these hearts have an increased heart rate in an isolated perfusion system. Since D2 is present in the human heart, we speculated that not only changes in serum thyroid hormone levels play an important role in the development of tachycardia and/or AF, but also genetic polymorphisms affecting the activity of D2.

**Methods:** We analyzed the association of the D2-ORFa-Gly3Asp and the D2-Thr92Ala polymorphisms in D2, and haplotypes thereof, and serum thyroid hormone levels with AF and with heart rate in a population based study of 1055 elderly subjects (72.2 ± 7.4 years, 45.8% males), of whom 28 subjects had AF.

**Results:** The minor allele frequencies of the D2-ORFa-Gly3Asp and D2-Thr92Ala polymorphisms were 34.8% (Asp3) and 37.5% (Ala92), respectively. No effect of either of the two polymorphisms was seen on serum thyroid hormone levels in this population. D2-ORFa-Asp3 allele carriers had an increased risk of AF (OR=3.10, 95%CI: 1.15-8.35, P=0.03), whereas D2-Ala92 allele carriers had a decreased risk of AF (OR=0.45, P=0.05), with evidence for allele-dose effects. The association became stronger when haplotypes were considered indicating additive effects of both risk alleles (i.e. Asp3/Thr92 (=haplotype allele 2) vs Gly3/Ala92 (=haplotype allele 3)). Carriers of the haplotype allele 2 had a 4.9 times higher risk of atrial fibrillation (95%CI: 1.38-17.64, P=0.01) than carriers of the haplotype 3 allele. No effect of those polymorphisms on pulse rate was observed, while FT4 and T3 were positively related with pulse rate (P<0.05). Subjects with AF, however, did not have higher levels of FT4 and T3.

**Conclusion:** D2 polymorphisms that result in an altered D2 activity influence the risk on AF.

## Introduction

The heart is one of the most sensitive organs to increased levels of thyroid hormone. Hyperthyroid patients have tachycardia and often atrial fibrillation (1, 2), and even patients with subclinical hyperthyroidism have a five-fold increased risk to develop atrial fibrillation (3). Thyroid hormone does not only have direct effects on the heart, affecting the expression of several important genes, but increased levels of thyroid hormone also result in an increased oxygen demand in the rest of the body, indirectly resulting in a higher cardiac workload (4, 5). Therefore, the effects

of thyroid hormone on the heart may be caused by both direct (intrinsic) and/or indirect (extrinsic) effects.

Production of thyroid hormone is regulated by the hypothalamus-pituitary-thyroid axis, whereas the biological activity of thyroid hormone, i.e. the availability of T3, is mainly regulated by the three selenodeiodinases (D1-D3) (1, 6, 7). Tissue distribution and enzymatic activity of the deiodinases differ, and they all play a different physiological role. D2 is present in brain, pituitary, thyroid, and brown adipose tissue (6, 7), and in humans, but not in rodents, D2 is also present in heart, skeletal muscle, and aortic smooth muscle cells (8-12). It converts T4 to T3 by outer ring deiodination, and is important in the regulation of local thyroid hormone bioactivity in D2-expressing tissues (7, 9), whereas D2 in thyroid and skeletal muscle may also contribute to serum T3 production (7, 9).

Recently, it was shown that overexpression of D2 in the mouse heart causes cardiac-specific thyrotoxicosis (13). Although plasma T4 and T3, as well as heart weight, were not affected by D2 overexpression, these hearts showed a significantly increased heart rate in an isolated perfusion system (13). Furthermore, it was shown that this chronic cardiac-specific thyrotoxicosis increases the β-adrenergic responsiveness of cardiomyocytes (14).

Based on these studies, we speculated that not only changes in serum thyroid hormone levels play an important role in the development of tachycardia and/or atrial fibrillation in humans, but also genetic variations affecting the activity of D2. Recently, two interesting polymorphisms in D2 have been identified, D2-ORFa-Gly3Asp (located in a short open reading frame in the 5'-UTR of D2) and D2-Thr92Ala (15-18). Based on its association with a higher T3/T4 ratio, the Asp3 allele of D2-ORFa-Gly3Asp seems to be associated with an increased activity of D2 (15), whereas the Ala92 allele of D2-Thr92Ala is associated with insulin resistance and a decreased D2 activity (16-18). Thusfar the D2-ORFa-Gly3Asp and D2-Thr92Ala polymorphisms have not been studied with respect to tachycardia and atrial fibrillation. We therefore assessed the association of these polymorphisms in D2, and haplotypes thereof, and serum thyroid hormone levels with heart rate and the development of atrial fibrillation in a population of over a thousand elderly subjects.

## *Research design and Methods*

### *Study population*

This study was based on participants in the Rotterdam Scan Study, a population based study which was designed to investigate determinants and consequences of

brain abnormalities on MRI in the elderly (19, 20). Subjects aged 60-90 years in strata of age and sex were randomly selected from the ongoing population-based Zoetermeer and Rotterdam studies (21, 22). As part of the eligibility criteria, subjects who were demented, blind or had MRI contraindications at time of selection were excluded. Complete information was obtained in 1077 subjects who gave written informed consent (participation rate 63%). The study was conducted in accordance with the tenets of the Declaration of Helsinki, and was approved by the Medical Ethics Committee of Erasmus Medical Center, The Netherlands.

Presence of atrial fibrillation was based on analysis of digitally stored standard 12-lead electrocardiograms using the Modular Electrocardiogram Analysis System (MEANS) (23). ECG was missing in four participants. Pulse rate was determined lying down (N=2) and in standing position (N=5). The mean of the different measurements was used for the analyses. Participants were asked to bring all prescribed drugs to the research center, where a physician checked the use. We included reported use of cardiac medication (including amiodarone), β-blocking agents, antihypertensives, diuretics, calcium-antagonists, drugs interacting with the Renine-Angiotensin-Aldosteron System (RAAS), lipid lowering drugs, and antithrombotics (Anatomical Therapeutical Chemical codes c01, c07, c02, c03, c08, c09, c10, and b01). Antihypertensives in the tables are defined as a combination of c02, c03, c07, c08, or c09. Blood pressure was measured twice on the right arm with a random-zero sphygmomanometer. Mean arterial blood pressure was defined as: ((2 X diastolic blood pressure + systolic blood pressure) / 3). Hypertension was defined as a systolic blood pressure $\geq$160mmHg, a diastolic blood pressure $\geq$95mmHg, or use of antihypertensives. Diabetes mellitus was defined as reported use of oral antidiabetic treatment or of insulin, or a non-fasting random or post-load serum glucose concentration > 11.1 mmol/l. Serum total and HDL cholesterol, creatinine, and glucose levels were determined using automated enzymatic procedures.

### *Serum analyses*

At time of MRI, non-fasting blood samples were collected and within 30 minutes serum was separated and stored at – 80° C. TSH, FT4 and T3 were measured by chemoluminescence assays (Vitros ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, Rochester, NY, USA). Reverse T3 (rT3) was measured with an in-house radioimmunoassay (24). Serum antibodies to thyroid peroxidase (TPO-Abs) were assessed with an immunometric assay (DPC, Los Angeles, CA, USA).

TSH and FT4 serum levels were used to define thyroid status. Hypothyroidism was defined as a concentration of serum TSH above the upper limit of the reference range (0.4-4.3 mU/l) and FT4 below the reference range (11-25 pmol/l). Hyperthyroidism was defined as a concentration of serum TSH below the reference range and FT4 above the reference range. Since thyroid hormone concentrations change with age, these reference values might be less appropriate in an elderly population (25). Therefore, we also analyzed the effects of serum thyroid hormone levels on pulse rate and atrial fibrillation according to the continuous distribution of the thyroid hormones in this population.

Evaluation of thyroid function in the elderly is complicated by an increased prevalence of non-thyroidal illness and autoimmune thyroid disease (25, 26). Several conditions such as malnutrition, starvation, inflammatory processes, and disease in general are known to alter TSH and thyroid hormone concentrations, in the absence of thyroid disease. In these situations, plasma T3 decreases and plasma rT3 increases, and the magnitude of these changes is related to the severity of the disease (7, 27-30). Serum rT3 levels (reference range 0.14 - 0.34 nmol/l and the ratio of T3 over rT3 (T3/rT3) were therefore considered to be markers of non-thyroidal illness (27, 30). Data on TSH, FT4, atrial fibrillation, and the use of either cardiac or thyroid medication were available of 1055 subjects.

### *Genotyping*

DNA was isolated from peripheral leucocytes by standard procedures, and was available of 1032 subjects. Genotyping was done by 5' fluoregenic Taqman assays and reactions were performed in 384-wells format on ABI9700 2x384well PCR machines with endpoint reading on the ABI 7900HT TaqMan® machine (Applied Biosystems, Nieuwerkerk aan den Ijssel, The Netherlands). Genotypes were determined using 5 ng of gDNA per SNP genotype by the Taqman allelic discrimination assay. Primer and probe sequences were optimized using the single nucleotide polymorphism assay-by-design service of Applied Biosystems. For details see: http://store.appliedbiosystems.com. Genotyping for the D2-Thr92Ala polymorphism failed in 4 subjects.

### *Statistical analysis*

Data were analyzed using SPSS 10.0.7 for Windows (SPSS Inc., Chicago, IL). Non-parametric Mann-Whitney-U tests were used to see if there was a difference in possible confounders between subjects with or without atrial fibrillation, or between carriers and non-carriers of the D2-ORFa-Gly3Asp and D2-Thr92Ala polymorphisms.

These confounders were also analyzed by linear or logistic regression, adjusted for age and sex. Differences between the genotype groups were adjusted for age and tested by analysis of covariance (ANCOVA) using the general linear model procedure. In case of an allele dose effect, we performed a (multiple) linear regression analysis to quantify the association. The effects of the polymorphisms on serum indices of thyroid function were analyzed after the exclusion of the subjects on thyroid hormone treatment and / or with a TSH outside the reference range (n=131). The association of the polymorphisms with atrial fibrillation was analyzed by logistic regression, and adjusted for age and gender. These analyses were repeated after exclusion of all subjects with biochemically defined hypo- (n = 4) or hyperthyroidism (n = 13), and / or those using thyromimetic or thyrostatic medication (n=23), and also after the additional exclusion of subjects who were using cardiac medication. Deviation from Hardy-Weinberg equilibrium was analyzed using a Chi-square test. Haplotype allele frequencies were estimated using the PHASE program (version 2) (31).

Multivariate linear regression models were used to quantify the relation between thyroid function and pulse rate. All analyses were adjusted for age and sex. In addition, these analyses were repeated after exclusion of subjects with biochemically hypo- or hyperthyroidism, and / or those using thyromimetic or thyrostatic medication, and also after the additional exclusion of subjects who were using cardiac medication. Assumptions of the model were verified by residual diagnostics. Differences in serum thyroid parameters between the subjects with and without atrial fibrillation were adjusted for age and tested by ANCOVA. P values are two-sided throughout, and $P<0.05$ was considered significant.

## Results

### *Descriptive statistics of the population*

Table 1 shows the descriptive statistics of the study population. Based on serum TSH and FT4 levels, 0.4% of this population was hypothyroid, and 1.2% was hyperthyroid. Six of the 13 hyperthyroid patients used Levothyroxine, compared to none of the 4 patients with hypothyroid values. Subjects who had atrial fibrillation were older, used more often cardiac medication, and used more often antithrombotics, had a higher pulse rate.

**Table 1.** Descriptive statistics of the population of the subjects with and without atrial fibrillation

| | | Atrial Fibrillation | | |
|---|---|---|---|---|
| | N=1055 | No (n=1027) | Yes (n=28) | *P* |
| Age (years) | 72.1 ± 7.4 | 72.0 ± 7.3 | 77.4 ± 6.9 | |
| No. males [%] | 511 [48.4 %] | 494 [48.1 %] | 17 [60.7 %] | |
| BMI (kg/m2) | 26.7 ± 3.6[a] | 26.7 ± 3.6a | 26.8 ± 3.1 | 0.70 |
| TSH (mU/L) | 1.15 (0.72 – 1.76) | 1.15 (0.73 – 1.74)[g] | 1.50 (0.74 – 2.22)[g] | **0.04*** |
| FT4 (pmol/L) | 18.1 ± 3.4 | 17.9 ± 3.0[g] | 18.8 ± 3.0g | 0.35* |
| T3 (nmol/L) | 2.01 ± 0.42 | 2.02 ± 0.42[g] | 1.88 ± 0.27[g] | 0.15* |
| rT3 (nmol/L) | 0.35 ± 0.12[b] | 0.34 ± 0.11[b] | 0.39 ± 0.15[b] | 0.42* |
| T3/rT3 (Molar ratio) | 6.31 ± 2.07 [b] | 6.37 ± 1.99[b] | 5.37 ± 1.70[b] | 0.15* |
| No. receiving thyroid medication[%] | 23 [2.2%] | 22 [2.1%] | 1 [3.6%] | 0.39 |
| Hypertension | 548 [51.9%] | 529 [51.5%] | 19 [67.9%] | 0.26 |
| Systolic Bloodpressure | 147.3 ± 21.6[c] | 147.4 ± 21.6[c] | 142.7 ± 21.5 | 0.07 |
| Diastolic Bloodpressure | 78.7 ± 11.8[c] | 78.7 ± 11.8[c] | 76.5 ± 10.9 | 0.58 |
| Pulse rate (lying down) | 67.4 ± 11.0[d] | 67.1 ± 10.7[d] | 79.8 ± 15.6[d] | **<0.001** |
| Pulse rate (standing) | 75.7 ± 12.1[e] | 75.6 ± 12.1[e] | 80.8 ± 9.4[e] | 0.02 |
| Myocardial infarction | 82 [7.8%] | 81 [7.9%] | 1 [3.6%] | 0.27 |
| No. receiving cardiac medication[%] | 105 [10.0%] | 91 [8.9%] | 14 [50.0%] | **<0.001** |
| Antihypertensives | 366 [34.7%] | 349 [34.0%] | 17 [60.7%] | 0.80 |
| Anti trombotics | 195 [18.5%] | 182 [17.7%] | 13 [46.4%] | **<0.001** |
| Diabetes Mellitus | 74 [7.0%][f] | 69 [6.7%][f] | 5 [17.9%] | 0.08 |
| Glucose | 6.1 ± 1.8[f] | 6.1 ± 1.8[f] | 6.8 ± 2.7 | 0.10 |
| Cholesterol | 5.9 ± 1.0 | 5.9 ± 1.0 | 5.5 ± 0.9 | 0.13 |
| HDL-C | 1.28 ± 0.35 | 1.29 ± 0.35 | 1.14 ± 0.27 | 0.06 |
| Lipid lowering drugs | 73 [6.9%] | 73 [7.1%] | 0 [0%] | 0.74 |
| Creatinine | 88.8 ± 18.5[f] | 88.6 ± 18.4[f] | 96.7 ± 21.2 | 0.27 |

Data are shown as mean ± SD, as median [IQR], or as no. [%]. IQR, Interquartile range; P values are adjusted for age and gender. All subjects on thyroid medication and/or who were biochemically hyper- or hypothyroid (n=33) were excluded from the analysis
[a] N= 1053, and 1025, respectively [b] N= 1040, 981, and 27 [c] N= 1049, and 1021 [d] N= 1040, 1014, and 26 [e] N= 1014, 993, and 21, [f] N= 1054, and 1026, [g] N= 995, and 27

The allele frequency of the D2-ORFa-Asp3 allele was 34.6 %, and the frequency of the D2-Ala92 allele was 34.6 %, and the frequency of the D2-Thr92Ala polymorphism was 37.6 %. Genotype distributions followed Hardy Weinberg equilibrium. No significant difference was found in any of these variables between carriers and non-carriers of either the D2-ORFa-Gly3Asp or the D2-Thr92Ala polymorphism (data not shown), but carriers of the D2-ORFa-Asp3 allele tended to be more often males (50.4 % vs 44.5 % in non-carriers, P=0.06) and more often to use β-blockers 16.5 % vs 13.0 %, P=0.12), whereas carriers of the D2-Ala92 allele tended to use β-blockers less frequently (13.6 % vs 17.5 %, P=0.09).

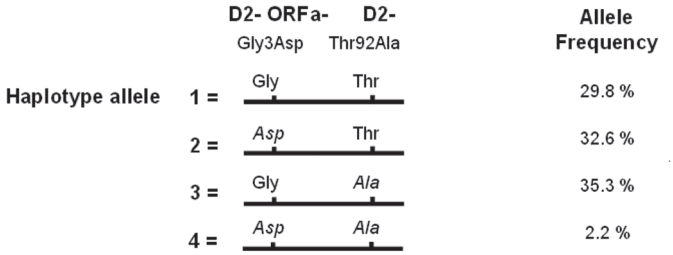


**Figure 1.** Four haplotype alles that were constructed from the D2-ORFa-Gly3Asp and the D2-Thr92Ala polymorphisms. Frequency estimates of the haplotype alleles in the studied population are shown.

No effect of either of the two polymorphisms was seen on serum thyroid hormone levels (Table 2). D2-ORFa-Gly3Asp and D2-Thr92Ala were analyzed for linkage disequilibrium (D'=0.88) using the PHASE program (31), and haplotype allele frequencies were estimated and are shown in Fig. 1 The haplotype allele with both the D2-ORFa-Asp3 and D2-Ala92 variant (haplotype allele 4) was present at a low frequency (2.2 %), indicating that the variant alleles appear predominantly on separate (frequent) haplotype alleles. The analysis of haplotype allele 2 carriers vs. the rest of the population gave therefore similar results as the analysis of carriers the D2-ORFa-Asp3 variant. Similarly, the association analysis of haplotype allele 3 in this population gave similar results as the association analysis of the D2-Ala92 variant.

**Table 2.** Plasma TSH and iodothyronine levels by D2 genotype

| | **D2-ORFa-Gly3Asp** | | | **D2-Thr92Ala** | | |
|---|---|---|---|---|---|---|
| | Gly/Gly | Gly/Asp + Asp/Asp | ***P*** | Thr/Thr | Thr/Ala + Ala/Ala | ***P*** |
| N | 383 | 522 | | 358 | 544 | |
| TSH (mU/L) | 1.40 ± 0.04 | 1.39 ± 0.03 | 0.68 | 1.35 ± 0.04 | 1.42 ± 0.03 | 0.43 |
| FT4 (pmol/L) | 17.8 ± 0.2 | 18.0 ± 0.1 | 0.54 | 17.9 ± 0.16 | 18.0 ± 0.13 | 0.60 |
| T3 (nmol/L) | 2.02 ± 0.02 | 1.98 ± 0.02 | 0.12 | 1.99 ± 0.02 | 2.01 ± 0.02 | 0.60 |
| RT3 (nmol/L) | 0.34 ± 0.01[a] | 0.35 ± 0.01[a] | 0.57 | 0.34 ± 0.01[b] | 0.35 ± 0.01[b] | 0.66 |
| TSH/FT4 | 0.083 ± 0.003 | 0.081 ± 0.002 | 0.57 | 0.079 ± 0.003 | 0.083 ± 0.002 | 0.56 |

[a] N=379 vs 515, [b] N=354 vs. 537

Data are adjusted for age and gender

We excluded all subjects on thyroid medication and/or with a TSH outside the reference range (n=131).

*The relation between D2 polymorphisms and pulse rate and atrial fibrillation*

Carriers of the D2-ORFa-Asp3 allele had an increased risk of atrial fibrillation compared to non-carriers (OR=2.61, 95% CI: 1.04-6.56, P=0.04), with evidence for an allele-dose effect (Table 3). Exclusion of all subjects on thyroid medication and/or those who were biochemically hyper- or hypothyroid increased the effect (OR=3.10, 95% CI: 1.15-8.35, P=0.03) (Table 3). On the other hand, carriers of the D2-Ala92 allele showed a tendency towards a decreased risk of atrial fibrillation compared to non-carriers (OR=0.49, P=0.07), with also evidence for an allele-dose effect (Table 3). This decreased risk of atrial fibrillation in carriers of the D2-Ala92 allele was significant after the exclusion of all subjects on thyroid medication and/or who were biochemically hyper- or hypothyroid (OR=0.45, P=0.05) (Table 3).

**Table 3.** Risk of atrial fibrillation by D2 genotype

| | D2-ORFa-Gly3Asp | *P* | D2 Thr92Ala | *P* | Haplotype allele 2[c] | *P* |
|---|---|---|---|---|---|---|
| All subjects[a] | | | | | | |
| Wildtypes | 1 | | 1 | | 1 | |
| Heterozygotes | 2.41 (0.92; 6.30) | 0.07 | 0.52 (0.23; 1.18) | 0.12 | | |
| Homozygotes | 3.33 (1.04; 10.7) | **0.04** | 0.38 (0.08; 1.69) | 0.20 | | |
| Carriers | 2.61 (1.04; 6.56) | **0.04** | 0.49 (0.23; 1.06) | 0.07 | 3.78 (1.21; 11.81) | **0.02** |
| After exclusion*[b] | | | | | | |
| Wildtypes | 1 | | 1 | | 1 | |
| Heterozygotes | 2.85 (1.02; 7.96) | **0.05** | 0.48 (0.21 ; 1.11) | 0.09 | | |
| Homozygotes | 4.00 (1.18; 13.56) | **0.03** | 0.37 (0.08; 1.66) | 0.19 | | |
| Carriers | 3.10 (1.15; 8.35) | **0.03** | 0.45 (0.20; 1.01) | **0.05** | 4.94 (1.38; 17.64) | **0.01** |

* After the exclusion of all subjects on thyroid medication and/or were biochemically hyper- or hypothyroid

Odds ratios are adjusted for age and gender.

[a] 27 subjects out of 1028 had atrial fibrillation, [b] 26 subjects out of 999 had atrial fibrillation.

[c] The analysis of carriers of haplotype 2 vs carriers of haplotype 3, after the exclusion of all subjects who carried none of these alleles and of all subjects who carried a combination of allele 2 and allele 3 (N=380).

The association became stronger when haplotypes were considered indicating an additive effect of both risk alleleles (i.e. Asp3 and Thr92 (=haplotype allele 2) vs Gly3 and Ala92 (=haplotype allele 3)). Analysis of carriers of the haplotype 2 allele versus carriers of the haplotype 3 allele, by exclusion of all subjects who carried none of these alleles and of all subjects who carried a combination of allele 2 and allele 3 (N=380), showed that carriers of the haplotype allele 2 had a 3.8 times higher risk of atrial fibrillation (95% CI :1.21 to 11.81, P=0.02) than carriers of the haplotype 3 allele. Exclusion of all subjects on thyroid medication and/or those who were biochemically hyper- or hypothyroid made this effect stronger (OR=4.94, 95% CI: 1.38 to 17.64, P=0.01) (Table 3). Analysis of only homozygous carriers of haplotype allele 2 versus haplotype allele 3 gave similar results, which failed to reach significance due to a decreased power (data not shown).

Although the polymorphisms in D2 were associated with atrial fibrillation in this population, carriers of the D2-ORFa-Asp3 allele and of the D2-Ala92 allele, had similar pulse rates compared to non-carriers of these alleles (Table 4), regardless of whether subjects on thyroid medication and/or who were biochemically hyper- or hypothyroid, or who used β-blockers or Ca-antagonists were excluded or not.

**Table 4.** Pulse rate by D2 genotype

| | **D2ORFa-Gly3Asp** | | | **D2-Thr92Ala** | | |
|---|---|---|---|---|---|---|
| | Gly/Gly | Gly/Asp + Asp/Asp | ***P*** | Thr/Thr | Thr/Ala + Ala/Ala | ***P*** |
| N | 424 | 569 | | 379 | 602 | |
| Pulse rate lying down | 68.0 ± 0.5 | 67.2 ± 0.5 | 0.25 | 67.3 ± 0.6 | 67.7 ± 0.5 | 0.57 |
| Pulse rate standing | 76.3 ± 0.6 | 75.3 ± 0.5 | 0.17 | 75.2 ± 0.6 | 76.1 ± 0.5 | 0.27 |

All subjects on thyroid medication and/or who were biochemically hyper- or hypothyroid were excluded from the analysis. Additional exclusion of all subjects who used B-blockers and or Ca- antagonists did not alter the results. Data are adjusted for age and gender.

## *The relation between serum thyroid parameters and pulse rate and atrial fibrillation*

Subjects who had FT4 or T3 levels above the median had a significantly higher pulse rate (lying down as well as standing) than subjects who had FT4 or T3 levels below the median (P= 0.01 for FT4, and P<0.01 for T3, respectively). In subjects with a TSH above the median, the standing pulse rate tended to be lower (P=0.07). Linear regression analysis showed a significant positive effect of both FT4 and T3 on pulse

rate (lying down as well as standing), whereas serum TSH had a negative effect on the standing pulse rate (Table 5). Data shown in this table are after the exclusion of all subjects on thyroid medication, and after the exclusion of all subjects who were biochemically hyper- or hypothyroid. Additional exclusion of all subjects who used cardiac medication, or analysis of the entire population without any exclusion, gave similar results (data not shown).

**Table 5.** Association between serum thyroid parameters and pulse rate

| | **Pulse lying down** | | **Pulse standing** | |
|---|---|---|---|---|
| | Estimate (95% CI) | ***P*** | Estimate (95% CI) | ***P*** |
| Serum TSH (mU/L) | -0.53 (-1.29 to 0.23) | 0.17 | -0.95 (-1.79 to –0.10) | **0.03** |
| Serum FT4 (nmol/L) | 0.27 (0.05 to 0.50) | **0.02** | 0.29 (0.04 to 0.54) | **0.03** |
| Serum T3 (nmol/L) | 1.89 (0.27 to 3.50) | **0.02** | 2.18 (0.40 to 3.96) | **0.02** |
| Serum rT3 (nmol/L) | 1.15 (-5.37 to 7.67) | 0.73 | -0.19 (-7.33 to 6.95) | 0.96 |

Values are age and gender adjusted regression coefficients per SD increase in pulse rate.(with 95% CI and P value). All subjects on TH medication, and/or who were biochemically hyper- or hypothyroid were excluded from the analysis (n=17).

Surprisingly, subjects with atrial fibrillation had a significantly higher TSH, whereas FT4, T3, rT3, and also the T3/rT3 ratio were not different between the two groups (Table 1). Data shown in this table are after the exclusion of subjects on thyroid medication, and after the exclusion of all subjects who were biochemically hyper- or hypothyroid. Analysis of the entire population gave similar results (data not shown).

Eighty-one subjects in this population met the criteria of subclinical hyperthyroidism (TSH < 0.4 mU/L and 11< FT4 < 25 pmol/L), two of them used thyroid medication. In subjects with subclinical hyperthyroidism TSH was lower (0.20 ± 0.16 vs 1.53 ± 0.05), whereas FT4 and T3 were significantly higher (19.0 ± 0.4 vs 18.0 ± 0.1, P=0.008 and 2.17 ± 0.05 vs 2.00 ± 0.01 nmol/L, P=0.001). Reverse T3 tended to be higher (P=0.06) and the T3/rT3 ratio was not different between subjects with and without subclinical hyperthyroidism. One of the 81 subjects with subclinical hyperthyroidism had atrial fibrillation, and although average pulse rate was higher in subjects with subclinical hyperthyroidism, the difference was far from reaching significance (P=0.99 and P=0.36 for lying and standing pulse rate respectively).

## Discussion

In this elderly population, the D2-ORFa-Gly3Asp and D2-Thr92Ala polymorphisms were associated with an altered risk of atrial fibrillation, but not with differences in serum thyroid parameters or pulse rate. Higher levels of FT4 and T3 were associated with a higher pulse rate, but not with atrial fibrillation.

The Asp-allele of the D2-ORFa-Gly3Asp polymorphism and the Thr-allele of the D2-Thr92Ala polymorphism seem to be associated with an increased activity of D2 (15-18). Allele frequencies of these polymorphisms were in agreement with previous data in other populations (15-18). These alleles occur together on haplotype allele 2 and are both absent on haplotype allele 3. Based on the observation that overexpression of D2 in the mouse heart causes cardiac-specific thyrotoxicosis (13), and since atrial fibrillation is a frequent complication of hyperthyroidism (1, 2), an effect of these polymorphisms on atrial fibrillation might be expected. Indeed, carriers of the haplotype allele 2 had a ~4-fold increased risk of atrial fibrillation compared to carriers of the haplotype allele 3.

It is known for many years that hyperthyroidism is a risk factor for atrial fibrillation (1, 2), and more and more evidence becomes available that subclinical hyperthyroidism is also associated with atrial fibrillation (3, 32-34). The suppressed TSH in subjects with subclinical hyperthyroidism may thus indicate an excess of thyroid hormone, although FT4 levels are within the normal range, suggesting a unique set-point for each individual (35, 36). Genetic variations, and thus polymorphisms, are important determinants in the inter-individual variation in these serum thyroid function tests (17, 37, 38). Similarly, equal serum FT4 levels may result in higher concentrations of T3 in the heart of carriers of the haplotype 2 allele than in carriers of the haplotype 3 allele, resulting in a higher risk of developing atrial fibrillation.

In relatively young patients with subclinical hyperthyroidism, an increased left ventricular mass, enhanced resting systolic function and significantly impaired parameters of diastolic function have been described (39-41). Unfortunately, no echocardiographic data were available of the subjects in this study, since it would be interesting to know if these polymorphisms are also associated with an altered systolic or diastolic function of the heart.

In agreement with previous studies, no effect of either of the two polymorphisms was seen on circulating thyroid hormone levels in this elderly population (15, 17, 18), which obviously does not exclude an effect on local thyroid hormone bioactivity. Because overexpression of D2 in the mouse heart also results in an increased heart rate (13), and since patients with hyperthyroidism nearly always have tachycardia (1, 2), an association of the D2-ORFa-Gly3Asp and D2-Thr92Ala

polymorphisms with pulse rate would be expected. However, this was not the case. The observation that these polymorphisms are associated with atrial fibrillation but not with pulse rate may be explained in several ways. First, it might be due to a selection bias. Carriers of the D2-ORFa-Asp3 allele tended to use β-blockers more often, whereas carriers of the D2-Ala92 allele tended to use β-blockers less frequently. This, and perhaps other (cardiac) medication, may have masked possible effects of these polymorphisms on heart rate. However, adjustment for the use of medication (and specifically for β-blockers) did not affect the lack of association between the polymorphisms and pulse rate. Second, it may be due to different etiological mechanisms and/or the distribution of D2 in the heart. The effects of thyroid hormone on atrial fibrillation are probably mediated by an enhanced atrial excitability and a shortened refractory period of the conducting tissue (39, 41), whereas the inotropic effects of thyroid hormone are probably initiated in the SA node and mediated by the autonomous nervous system (42-44). The expression pattern of D2 in the human heart is unknown, and it is unclear if D2 is expressed in SA node at all. Third, the two polymorphisms in D2 might be linked to other polymorphisms that affect atrial fibrillation but not heart rate via a completely different mechanism. Current databases do not provide any evidence for this hypothesis.

As expected, FT4 and T3 were positively associated with pulse rate. Subjects with atrial fibrillation, however, did not have higher levels of FT4 and T3, and even had a higher TSH. This might be due to a mild form of non-thyroidal illness in patients with atrial fibrillation. Atrial fibrillation is a serious condition, and these patients used significantly more medication. This may very well have influenced the thyroid hormone levels of the subjects with atrial fibrillation. Nevertheless, serum rT3 and the T3/rT3 ratio, which are sensitive parameters of non-thyroidal illness, also in the elderly (27, 30, 45), were not different between subjects with and without atrial fibrillation.

In conclusion, polymorphisms that are associated with a higher activity of D2 result in an increased risk of having atrial fibrillation in this population of elderly males and females. These polymorphisms may contribute to explain why some people with subclinical hyperthyroidism do develop atrial fibrillation, whereas others with hyperthyroidism do not.

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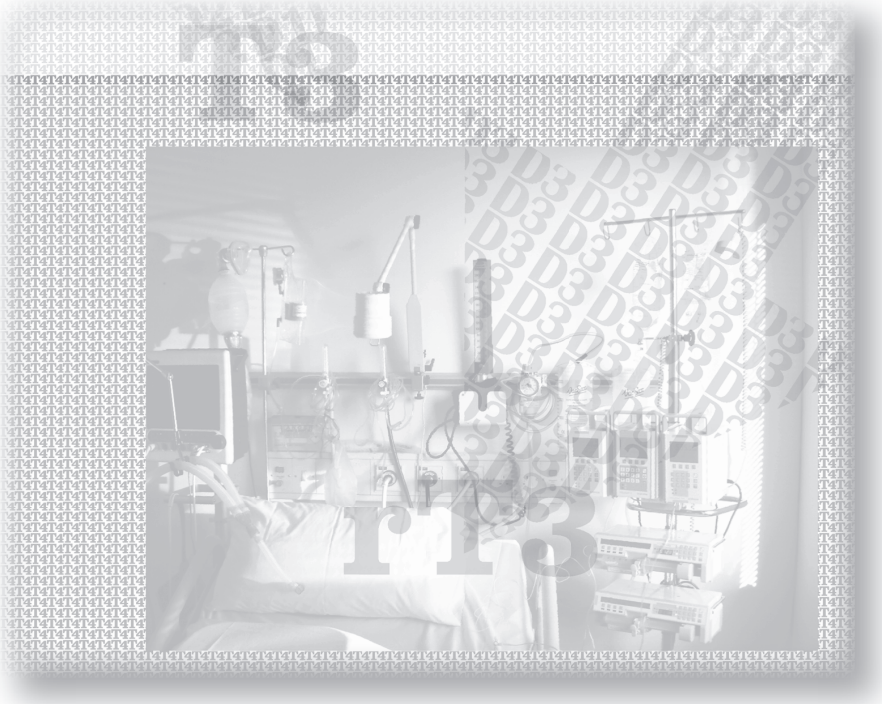
# *Part B*

## Regulation of thyroid hormone bioactivity during critical illness



# *Chapter B1*

## Reduced activation and increased inactivation of thyroid hormone in tissues of critically ill patients

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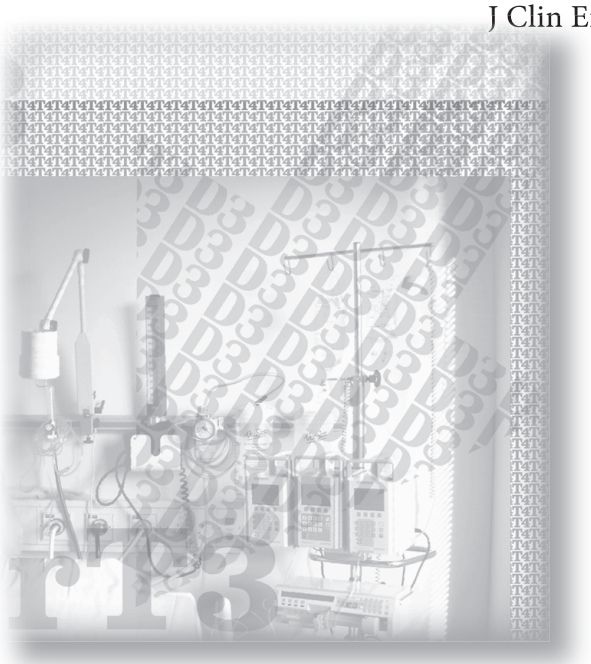
J Clin Endocrinol Metab. 88:3202-11



## Abstract

**Introduction**: Critical illness is often associated with reduced TSH and thyroid hormone secretion as well as with marked changes in peripheral thyroid hormone metabolism, resulting in low serum T3 and high rT3 levels. To study the mechanism(s) of the latter changes, we determined serum thyroid hormone levels and the expression of the type 1, 2, and 3 iodothyronine deiodinases (D1, D2 and D3) in liver and skeletal muscle from deceased intensive care patients.

**Methods:** To study mechanisms underlying these changes, 65 blood samples, 65 liver and 66 skeletal muscle biopsies were obtained within minutes after death from 80 ICU patients, randomized for intensive or conventional insulin treatment. Serum thyroid parameters and the expression of tissue type 1, 2, and 3 iodothyronine deiodinases (D1-D3) were determined.

**Results:** Serum TSH, T4, T3, and the T3/rT3 ratio were lower, whereas serum rT3 was higher than in normal subjects ($P<0.0001$). Liver D1 activity was downregulated and D3 activity was induced. Serum T3/rT3 ratio correlated positively with liver D1 activity ($P<0.001$), and negatively with liver D3 activity (ns). These parameters were independent of the type of insulin treatment. Liver D1 and serum T3/rT3 were highest in patients who died from severe brain damage, intermediate in those who died from sepsis or excessive inflammation and were lowest in patients who died from cardiovascular collapse ($P<0.01$). Liver D3 showed an opposite relationship. Acute renal failure requiring dialysis and need of inotropes were associated with low liver D1 activity ($P<0.01$ and $P=0.06$) and high liver D3 ($P<0.01$) and skeletal muscle D3 ($P<0.05$) activity. Liver D1 activity was negatively correlated with plasma urea ($P=0.002$), creatinine ($P=0.06$) and bilirubin ($P<0.0001$). D1 and D3 mRNA levels corresponded with enzyme activities (both $P<0.001$), suggesting regulation of the expression of both deiodinases at the pretranslational level.

**Conclusion:** This is the first study relating tissue deiodinase activities with serum thyroid hormone levels and clinical parameters in a large group of critically ill patients. Liver D1 is downregulated and D3 (which is not present in liver and skeletal muscle of healthy individuals) is induced, particularly in disease states associated with poor tissue perfusion. These observed changes, in correlation with a low T3/rT3 ratio, may represent tissue-specific ways to reduce thyroid hormone bioactivity during cellular hypoxia and contribute to the "low T3 syndrome" of severe illness.

## Introduction

Critical illness induces a variety of hormonal changes which differ between acute and prolonged critical illness (1). Striking alterations observed in acute critical illness include increased serum catecholamines and cortisol levels, a blunted growth hormone pulsatility, insulin resistance and the so-called low T3 syndrome (1, 2). In prolonged critical illness, catecholamine and cortisol levels decrease compared to the acute situation, and a further decrease in the levels of growth hormone, TSH and thyroid hormone occurs. There are no indications that the acute changes are harmful, but it is unclear if the endocrine changes in prolonged critical illness are all beneficial adaptations since recent data suggest that some may contribute to a worsening of the clinical condition (3).

Substitution of critically ill patients with high doses of growth hormone, corticosteroids, or thyroid hormone has been found to have no or even a negative effect on clinical outcome (4-7). In a very recent study it was shown that strict control of blood glucose levels below 110 mg/dl with intensive insulin therapy markedly reduces morbidity and mortality in critically ill patients (8). Endocrine intervention with hypothalamic releasing factors, which restores pulsatile pituitary hormone secretion and normalizes peripheral hormone levels, may be another successful approach (3, 9).

In this study we focused on the peripheral metabolism of thyroid hormone in critically ill patients. In critical illness, serum 3,5,3'-triiodothyronine (T3) concentration decreases and serum 3,3',5'-triiodothyronine (reverse T3 or rT3) increases, the magnitudes of these changes being related to the severity of disease (10). Although serum thyroxine (T4) and free T4 (FT4) may be increased in mild illnesses, serum T4 is decreased and FT4 normal or decreased in severely ill patients (10).

In humans, peripheral thyroid hormone metabolism is mediated importantly by the three iodothyronine deiodinases D1, D2 and D3 (see Refs 11 & 12 for reviews). D1 is present in liver, kidney, and thyroid, and plays a key-role in the production of serum T3 from T4 and in the breakdown of the metabolite rT3 (11, 12) D2 is present in brain, pituitary, thyroid, and skeletal muscle, and also converts T4 by outer ring deiodination to T3. In tissues such as the brain, D2 is important for local T3 production, but the enzyme in skeletal muscle may also contribute to plasma T3 production (11, 12). D3 is present in brain, skin, placenta, pregnant uterus, and various fetal tissues; it catalyzes the inactivation of T4 and T3 by inner ring deiodination to rT3 and 3,3'-diiodothyronine (3,3'-T2), respectively (11, 12).

The strongly reduced circulating T3 levels in sick patients may in part be due to a decreased peripheral T4 deiodination by D1, D2, or both (12-14). The increase

in serum rT3 levels is explained by a decrease in D1 activity, since D1 is the principal pathway for rT3 clearance (15). Besides a decreased D1 activity, an impaired transport of T4 and rT3 into D1-containing tissues such as liver may be another important mechanism for the changes in thyroid hormone levels associated with illness (16). However, the possibility that an increased D3 activity contributes to the reduced serum T3 levels and increased rT3 levels should also be considered. Patients with D3-expressing hemangiomas may have very low serum T4 and T3 concentrations, combined with very high serum rT3 levels (17). The term 'consumptive hypothyroidism' is used for this syndrome. There are hitherto no data on the eventual role of D3 induction in the low T3 syndrome of severe illness.

In this study, serum samples and liver and skeletal muscle biopsies were obtained from 80 patients within minutes after they died in a surgical intensive care unit (ICU). The patients had been randomized for intensive or conventional insulin therapy as recently described (8). Tissue D1, D2 and D3 gene expression and activity as well as serum TSH, T4, T3 and rT3 concentrations were measured. The correlations between these analytes as well as their correlations with several clinical parameters were calculated.

## Materials and Methods

### *Materials*

Nonradioactive iodothyronines were obtained from Henning (Berlin, Germany). [3',5'-$^{125}$I]T4 (with a specific activity of ca. 2000 mCi/μmol) was obtained from Amersham Pharmacia (Rozendaal, The Netherlands). [3'-$^{125}$I]T3 and [3',5'-$^{125}$I]rT3 (with both a specific activity of ca. 2000 mCi/μmol) were prepared by radioiodination of 3,5-T2 and 3,3'-T2, respectively (18). [$^{125}$I]T4 and [$^{125}$I]rT3 were purified immediately before use by Sephadex LH-20 (Amersham Pharmacia) chromatography (19). N-bromoacetyl-[$^{125}$I]T3 (BrAc[$^{125}$I]T3) was prepared as previously described (20). Its purity was checked by HPLC analysis. Protein molecular weight markers, and 6-n-propyl-2-thiouracil (PTU) were obtained from Sigma (Zwijndrecht, The Netherlands); dithiothreitol (DTT) from ICN (Zoetermeer, The Netherlands); electrophoresis grade SDS-PAGE reagents from Bio-Rad (Veenendaal, The Netherlands); Coomassie Brilliant Blue R-250 from Merck (Darmstadt, Germany); and TaqMan$^{TM}$ probes and primers from Biosource (Nivelles, Belgium). All other chemicals used in this study were of reagent grade.

## *Subjects*

This study was part of a large randomized controlled study on intensive insulin treatment in ICU patients (N=1548), of which the major clinical outcomes have been published in detail elsewhere (8). On admission, patients were randomly assigned to either strict normalization of blood glucose (80-110 mg/dl) with intensive insulin therapy or the conventional approach, in which insulin infusion is initiated only when blood glucose exceeds 215 mg/dl, to maintain blood glucose levels between 180 and 200 mg/dl. Maximal insulin dose was arbitrarily set at 50IU/hour. The study protocol has been approved by the Ethical Review Board of the University of Leuven School of Medicine and patients were included after informed consent from the closest family member.

A total of 80 patients were included in this study. Blood samples were obtained from 65 patients, liver biopsies from 66 patients, and skeletal muscle (rectus abdominis) biopsies from 66 patients within minutes after death (25.2±20.0 (SD), range [5-97 min] for liver and 20.7±19.7, range [0-95 min] for skeletal muscle). From 51 patients blood, liver as well as skeletal muscle samples were available. All patients had been randomized for conventional or intensive insulin treatment (8). 31 patients had been treated with thyroid hormone during the course of their critical illness when they had a serum T4 concentration below 50 nmol/l in the face of a normal thyroxine-binding globulin and concomitantly clinical symptoms of hypothyroidism, defined as coma or central nervous system suppression, failure to wean from the ventilator, or hemodynamic instability, which were unexplained and resistant to conventional supportive therapy. In these cases, thyroid hormone treatment consisted of an IV bolus of 150 μg T4 daily plus 0.6 μg T3 per kg body weight per 24 h as a continuous IV infusion. All patients included in this study had died in the ICU, and the cause of death was determined both clinically by the attending ICU physician and by post-mortem examination. The pathologist was unaware of insulin treatment allocation. Relevant patients' characteristics are summarized in Table 1.

**Table 1.** Patients characteristics, divided in different groups based on cause of death.

| | Cause of Death | | | | |
|---|---|---|---|---|---|
| | I | II | III | IV | Total |
| Number of patients | 11 | 36 | 27 | 6 | 80 |
| Age (yr) | 66.6 ± 11.1 | 70.2 ± 12.4 | 68.7 ± 12.8 | 62.1 ± 9.0 | 68.6 ± 12.1 |
| BMI | 28.4 ± 6.1 | 24.9 ± 3.5 | 25.2 ± 5.4 | 23.6 ± 3.0 | 25.4 ± 4.7 |
| ICU stay (days) | 8.7 ± 7.3 | 31.4 ± 34.5 | 24.9 ± 25.4 | 6.2 ± 5.7 | 24.2 ± 28.9 |
| Male | 5 | 26 | 20 | 4 | 45 |
| Intensive Insuline Therapy | 6 | 6 | 11 | 2 | 25 |
| Thyroid hormone treatment | 4 | 15 | 12 | 0 | 31 |
| Renal Replacement Therapy | 4 | 20 | 14 | 2 | 40 |
| Inotropes treatment | 10 | 32 | 21 | 3 | 66 |

I = cardiovascular collapse, II = MOF sepsis, III = MOF SIRS, IV = severe brain damage
data represent mean ± SD

### *Serum analyses*

The care of patients in the ICU often comprises infusion of heparin, either systemically or locally to prevent clotting of vascular access, which substantially interferes with the assay used to quantify free concentrations of thyroid hormone (21). Therefore, we refrained from measuring serum FT4 and FT3 in this study. Serum total T4, total T3, and TSH were measured by chemoluminescence assays (Vitros ECi Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK). rT3 was measured by radioimmunoassay as previously described (22). Within assay coefficients of variation amounted to 4% for TSH, 2% for T4, 2% for T3, and 3-4% for rT3. Normal values for TSH, T4, T3, and rT3 were determined in 270 healthy individuals. Mean ± 2 SD was used as the normal range for T4, T3, and rT3, whereas the 95% confidence interval was used for TSH.

### *Deiodinase activities*

Human liver and skeletal muscle samples were homogenized on ice in 10 volumes of PE buffer (0.1 M phosphate, 2 mM EDTA, pH 7.2) using a Polytron (Kinematica AG, Lucerne, Switzerland). Homogenates were snap-frozen in aliquots and stored at -80 C until further analysis. Protein concentration was measured with the Bio-Rad Protein Assay using BSA as the standard following the manufacturer's instructions.

Liver D1 activities were determined as described earlier (23) by duplicate incubations of homogenates (10 μg protein) for 30 min at 37 C with 0.1 μM [3',5'-$^{125}$I]rT3 (100,000 cpm) in a final volume of 0.1 ml PED10 buffer (PE + 10 mM DTT). To validate the specificity of the D1 assay, some incubations were also carried out in the presence of 0.1 mM of the D1 inhibitor PTU or excess unlabeled rT3 (1 μM). Reactions were stopped by addition of 0.1 ml 5% (wt/vol) BSA in water on ice. The protein-bound iodothyronines were precipitated by addition of 0.5 ml ice-cold 10% (wt/vol) trichloroacetic acid in water. Following centrifugation, $^{125}I^-$ was isolated from the supernatant by chromatography on Sephadex LH-20 minicolumns (24). Skeletal muscle D1 activities were assayed similarly, using 200 μg of homogenate protein in a 60 min incubation.

Liver and skeletal muscle D2 activities were assayed as earlier described (25) by duplicate incubation of 200 μg of homogenate protein for 60 min at 37 C with 1 nM [3',5'-$^{125}$I]T4 (100,000 cpm) in a final volume of 0.1 ml PED25 buffer (PE + 25 mM DTT). The incubations were carried out in the presence of 0.1 μM unlabeled T3, to prevent inner ring deiodination of the labeled T4 substrate by D3, if present, and in the absence or presence of 0.1 μM unlabeled T4 which is sufficient to saturate D2. Deiodination of labeled T4 in the absence minus that in the presence of excess unlabeled T4 represents D2 activity. The further procedure for the quantitation of $^{125}I^-$ production was the same as described above for the D1 assay.

Tissue D3 activities were measured as described earlier (24) by duplicate incubation of liver (100 μg protein) or skeletal muscle (200 μg protein) homogenate for 60 min at 37 C with 1 nM [3'-$^{125}$I]T3 (200,000 cpm) in a final volume of 0.1 ml PED50 buffer (PE + 50 mM DTT). To validate the D3 assay, some incubations were also carried out in the presence of 10 or 100 nM unlabeled T3. Reactions were stopped by addition of 0.1 ml ice-cold methanol. After centrifugation, 0.15 ml of the supernatant was added to 0.1 ml 0.02 M ammonium acetate (pH 4), and 0.1 ml of the mixture was applied to a 4.6 x 250 mm Symmetry C18 column connected to an Alliance HPLC system (Waters, Etten-Leur, The Netherlands). The column was eluted with a lineair gradient of acetonitrile (28%-42% in 15 min) in 0.02 M ammonium acetate (pH 4.0) at a flow of 1.2 ml/min. The radioactivity in the eluate was measured on-line using a Radiomatic A-500 flow scintillation detector (Packard, Meriden, CT).

### *Affinity labeling*

Affinity labeling of liver D1 was done as previously described (23) in samples with low, intermediate, or high D1 activity. Briefly, homogenate (100 μg protein) was

incubated for 15 min at 37 C with 100,000 cpm (~25 fmol) BrAc[$^{125}$I]T3 in 75 µl PED1 buffer (PE + 1 mM DTT). To specifically block affinity-labeling of D1 protein, some incubations were carried out in the presence of 10 µM rT3 and 100 µM PTU (23). Labeling was stopped by the addition of SDS-loading buffer and by heating the mixture for 5 min at 80 C. Proteins (100 µg per lane) were separated by SDS-PAGE gel electrophoresis in a 12% resolving, 5% stacking gel (23). Gels were stained with Coomassie Brilliant Blue R-250, dried at 80 C under vacuum, and autoradiographed by exposure overnight at -80 C to Kodak Biomax Imaging Film (Eastman Kodak, Rochester, NY).

### *RNA isolation and reverse transcription*

RNA was isolated from liver samples using the High Pure RNA Tissue Kit (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's protocol. RNA concentrations were determined using the RiboGreen® RNA Quantitation Kit (Molecular Probes, Leiden, The Netherlands). All samples were diluted to 0.1 µg/µl, and 1 µg was used for cDNA synthesis using the TaqMan™ Reverse Transcription Kit (Roche Diagnostics).

### *Real-time RT- PCR*

D1 and D3 mRNA levels were determined in a set of liver samples with low, intermediate, and high deiodinase activities. The ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) was used, which employs TaqMan™ chemistry for highly accurate quantitation of mRNA levels. Sequences and concentrations of the primers and probes are given in Table 2. Hepatic D1 and D3 mRNA levels are expressed relative to those of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene. The GAPDH probe and primers were provided as pre-optimized control system (Applied Biosystems).

Reactions were done for 2 min at 50 C and for 10 min at 95 C, followed by 40 cycles of 15 s at 95 C and for 1 min at 60 C. According to the manufacturer's guidelines, data were expressed as Ct values, which represent the cycle number at which probe-derived dye absorbance reaches the calculated treshold value. Data are expressed as ΔCt, *i.e.* the Ct value of the target gene minus the Ct value of the housekeeping gene.

**Table 2.** Sequences and concentrations of primers and probes that were used for determination of D1 and D3 mRNA levels by quantitative real-time RT-PCR.

| Primers & Probes | Sequence | Concentration |
|---|---|---|
| D1 Forward | 5'-TTAGTTCCATAGCAGATTTTCTTGTCA-3' | 200 nM |
| D1 Reverse | 5'-CTGATGTCCATGTTGTTCTTAAAAGC-3' | 200 nM |
| D1 Probe | 5'-FAM-AGCCATCTGATGCATGTGCTTCTTCAATG-TAMRA-3' | 100 nM |
| D3 Forward | 5'-TTCCAGAGCCAGCACATCCT-3' | 200 nM |
| D3 Reverse | 5'-ACGTCGCGCTGGTACTTAGTG-3' | 200 nM |
| D3 Probe | 5'-FAM-TGCACCTGACCACCGTTCATGGC-TAMRA-3' | 200 nM |

### *Statistical analysis*

Data were analyzed using the statistical program SPSS 10.0.7 for Windows (SPSS, Chicago, IL). Logarithmic transformations were applied in order to normalize variables and to minimize the influence of outliers, when appropriate. All analyses were done on the whole group, as well as on subgroups treated or not treated with thyroid hormone. Data were analyzed using one-way ANOVA tests, with a post-hoc Fisher's least significant difference (LSD) test for multiple comparisons, Student's t-tests, Mann-Whitney U tests and linear regression analyses, when appropriate.

## Results

Compared with the normal ranges for our laboratory, serum TSH, T4, T3, levels and the T3/rT3 ratio were low, whereas mean serum rT3 was high ($P<0.0001$) (Table 3). Patients who were treated with thyroid hormone had higher serum T3 and lower TSH levels than patients who were not treated with thyroid hormone. Serum T4 and rT3 levels were not different between these groups. No significant correlation was observed between serum TSH, T4, T3, and rT3, and post-mortem time.

Significant D1 activities were measured in all liver samples, with a range of 0.36-17.5 pmol/min per mg protein. In the liver samples tested, deiodination of the substrate rT3 was completely blocked by addition of the D1-specific inhibitor PTU, and was largely saturated by increasing the rT3 concentration from 0.1 to 1.1 μM.

The approximate Km value for rT3 was 0.4 ± 0.1 μM (mean ± SD, n=4), which is in good agreement with previous data (11,12). Negligible D1 activities were observed in the skeletal muscle homogenates. D2 activities were undetectable in all liver and skeletal muscle biopsies.

**Table 3.** Descriptive statistics of thyroid hormone levels and deiodinase activities in this population, subdivided in a group not receiving and a group receiving TH treatment

| | TH treatment | mean | median | SD | range | normal values | *P* [a] |
|---|---|---|---|---|---|---|---|
| TSH | | 0.75 | 0.17 | 1.58 | 0.001 - 10.7 | 0.2-4.2 | <0.0001 |
| mU/l | no (N=39) | 1.12 | 0.61 | 1.91 | 0.003 - 10.7 | | |
| | yes (N=25) | 0.18 ** | 0.01 | 0.48 | 0.001 - 2.15 | | |
| T4 | | 46.3 | 40.6 | 28.8 | 5.4 - 121 | 58-128 | <0.0001 |
| nmol/l | no (N=39) | 45.2 | 38.8 | 30.5 | 5.4 - 121 | | |
| | yes (N=25) | 48.0 | 44.7 | 26.4 | 9.6 - 98.1 | | |
| T3 | | 1 | 0.98 | 0.79 | 0.41 - 4.71 | 1.43-2.51 | <0.0001 |
| nmol/l | no (N=39) | 1.01 | 0.78 | 0.76 | 0.41 - 4.71 | | |
| | yes (N=25) | 1.60** | 1.41 | 0.72 | 0.67 - 3.21 | | |
| rT3 | | 1.85 | 1.37 | 2.15 | 0.22 - 15.78 | 0.14-0.34 | <0.0001 |
| nmol/l | no (N=39) | 1.75 | 1.13 | 2.56 | 0.22 - 15.78 | | |
| | yes (N=25) | 1.99 | 1.85 | 1.31 | 0.41 - 5.44 | | |
| T3/rT3 | | 1.2 | 0.8 | 1.2 | 0.1 - 6.1 | 4.2-17.9 | <0.0001 |
| | no (N=39) | 1.2 | 0.7 | 1.3 | 0.18 - 6.13 | | |
| | yes (N=25) | 1.2 | 0.9 | 1.1 | 0.3 - 4.8 | | |
| Liver D1 | | 4.51 | 3.25 | 3.89 | 0.44 - 17.53 | | |
| (pmol/mg/min) | no (N=44) | 4.57 | 3.33 | 3.65 | 0.52 - 16.78 | | |
| | yes (N=21) | 4.40 | 2.55 | 4.43 | 0.44 - 17.53 | | |
| Liver D3 | | 1.04 | 0.60 | 1.43 | 0.144 - 9.15 | | |
| (fmol/mg/min) | no (N=44) | 0.97 | 0.51 | 1.19 | 0.15 - 5.60 | | |
| | yes (N=21) | 1.18 | 0.71 | 1.87 | 0.14 - 9.15 | | |
| Muscle D3 | | 0.23 | 0.14 | 0.29 | 0.06 - 1.65 | | |
| (fmol/mg/min) | no (N=43) | 0.26 | 0.13 | 0.32 | 0.06 - 1.65 | | |
| | yes (N=22) | 0.23 | 0.16 | 0.24 | 0.09 - 1.14 | | |

[a] P values represent serum thyroid parameters of this population compared to normal values used in our laboratory.
** P<0.01 vs untreated group
To convert values for T4 to μg/dl, divide by 12.87, to convert values for T3 and rT3 to ng/dl, divide by 0.0154

Significant D3 activities were detected in most tissue samples, with ranges of 0.3-9.2 and 0.1-1.7 fmol/min per mg protein in liver and skeletal muscle, respectively. In both tissues, D3 activity was progressively saturated by increasing the T3 concentration from 1 to 10 and 100 nM, providing approximate Km values of 3.6 ± 0.6 nM T3 in liver and 2.3 ± 0.6 nM T3 for skeletal muscle (mean ± SD, n=4). These

values are in close agreement with previous reports (11,12). There was a significant correlation between D3 expression in liver and skeletal muscle (R=0.54, P<0.001), but in some patients high expression in liver was observed with low expression in skeletal muscle and *vice versa*. No significant correlation was observed between deiodinase activities and post-mortem time.

Serum thyroid hormone levels and tissue deiodinase activities were not different between the patients who had received intensive or conventional insulin therapy (Table 4). This has been found in the whole group and separately in the groups of patients who did or did not receive thyroid hormone treatment.

**Table 4.** The effect of insuline treatment on serum thyroid parameters and deiodinase activities

| | Intensive insulin treatment | mean | median | SD | range | normal values | *P* [a] |
|---|---|---|---|---|---|---|---|
| TSH | | | | | | 0.2-4.2 | |
| (mU/l) | no (N=48) | 0.93 | 0.21 | 1.79 | 0.001 - 10.7 | | |
| | yes (N=16) | 0.23 | 0.05 | 0.30 | 0.002 - 0.792 | | 0.08 |
| T4 | | | | | | 58-128 | |
| (nmol/l) | no (N=48) | 25.32 | 43.05 | 28.04 | 5.4 - 121 | | |
| | yes (N=16) | 49.28 | 37.40 | 31.65 | 18.8 - 118 | | 0.66 |
| T3 | | | | | | 1.43-2.51 | |
| (nmol/l) | no (N=48) | 1.19 | 0.96 | 0.68 | 0.41 - 3.21 | | |
| | yes (N=16) | 1.40 | 1.14 | 1.08 | 0.51 - 4.71 | | 0.64 |
| rT3 | | | | | | 0.14-0.34 | |
| (nmol/l) | no (N=48) | 1.73 | 1.52 | 1.32 | 0.24 - 5.44 | | |
| | yes (N=16) | 2.19 | 1.37 | 3.72 | 0.22 - 15.78 | | 0.48 |
| T3/rT3 | | | | | | 4.2-17.9 | |
| (nmol/l) | no (N=48) | 1.21 | 0.72 | 1.28 | 0.18 - 6.13 | | |
| | yes (N=16) | 1.27 | 1.09 | 0.91 | 0.30 - 3.29 | | 0.34 |
| Liver D1 | | | | | | | |
| (pmol/mg/min) | no (N=43) | 4.46 | 3.25 | 4.12 | 0.52 - 17.53 | | |
| | yes (N=22) | 4.61 | 3.21 | 3.48 | 0.44 - 11.44 | | 0.73 |
| Liver D3 | | | | | | | |
| (fmol/mg/min) | no (N=43) | 1.18 | 0.60 | 1.72 | 0.15 - 9.15 | | |
| | yes (N=22) | 0.76 | 0.62 | 0.46 | 0.14 - 1.84 | | 0.93 |
| Muscle D3 | | | | | | | |
| (fmol/mg/min) | no (N=42) | 0.28 | 0.14 | 0.36 | 0.055 - 1.65 | | |
| | yes (N=23) | 0.15 | 0.13 | 0.07 | 0.06 - 0.30 | | 0.24 |

[a] *P* values represent intensive insulin treatment vs conventional insulin treatment,analyzed by Mann-Whitney U test.

To convert values for T4 to µg/dl, divide by 12.87. To convert values for T3 and rT3 to ng/dl, divide by 0.0154

All regression analyses were also performed on the whole group and separately on the groups of patients who did or did not receive thyroid hormone treatment. Unless mentioned specifically, similar correlations were observed in the treated and untreated groups. The serum T3/rT3 ratio showed a positive correlation with liver D1 activity (linear regression test, R=0.66, P<0.001) and a negative, insignificant correlation with liver D3 activity (P=0.17) (Fig. 1) Serum T3/rT3 was not correlated with muscle D3 activity.

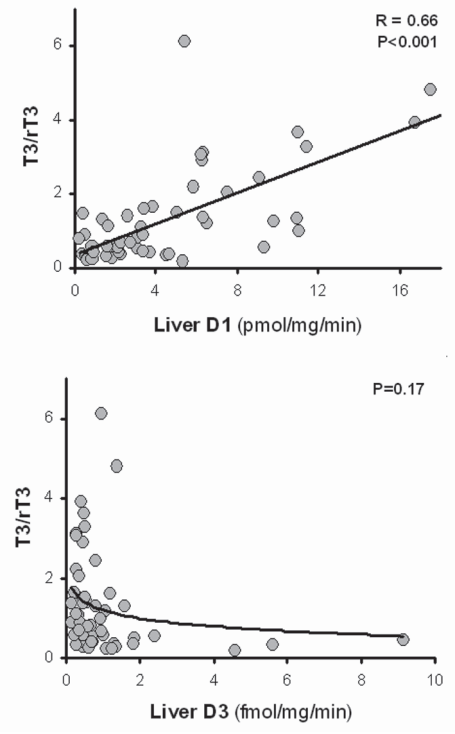



**Figure 1**. Correlation of liver D1 (A) and liver D3 (B) activities with the serum T3/rT3 ratio in 65 patients. No distinction is made in this figure between patients who received thyroid hormone treatment and patients who did not. Liver D1 shows a significant positive correlation with T3/rT3 ratio (P<0.001), whereas liver D3 shows an insignificant negative trend (P=0.17).

The possible relationships of tissue deiodinase activities with serum iodothyronine parameters other than the T3/rT3 ratio were also analyzed. Surprisingly, liver D1 activity showed a positive correlation with serum T4. This was due to the strong relationship in thyroid hormone-treated patients (R=0.74, P=0.002), whereas no correlation was seen in patients who had not received thyroid hormone (P=0.49). Liver D1 activity showed a negative correlation with serum rT3 (R=-0.48, P<0.001) and the serum rT3/T4 ratio (R=-0.55, P<0.001), independent of thyroid hormone treatment. Liver D1 activity was not correlated with serum T3 but showed an unexpected negative correlation with the serum T3/T4 ratio that was stronger in patients who had been treated *versus* those who had not been treated with thyroid hormone (R=-0.68, P=0.005 vs. R=-0.44, P=0.008).

Liver D3 activity showed a positive correlation with serum rT3 (R=0.40, P=0.016) and the rT3/T4 ratio (R=0.55, P=0.001) in the group of patients who had not been treated with thyroid hormone. These correlations were completely absent in the group of patients who had received thyroid hormone treatment. Skeletal muscle D3 activity showed a positive correlation with the serum rT3/T4 ratio (R=0.55, P=0.001). No other correlations were found for liver or skeletal muscle D3 activity with serum iodothyronine levels.

Hepatic D1 activity showed a significant correlation with cause of death, being lowest in the patients who had died of a cardiovascular collapse, with successive increases in the patients who had died of multiple organ failure (MOF) with sepsis or MOF with systemic inflammatory response syndrome (SIRS), and being highest in the patients who had died of severe brain damage (ANOVA P<0.01). Liver D1 activities in the latter group were similar to those determined in normal liver samples (data not shown). The serum T3/rT3 ratio showed a similar positive correlation with cause of death (ANOVA P<0.01), whereas liver D3 activity showed an insignificant negative correlation (ANOVA P=0.2) with cause of death. These relationships were strongest in the group of patients who had not been treated with thyroid hormone (Fig. 2), but similar correlations were also found in the group of patients who had been treated with thyroid hormone. D3 activities in skeletal muscle did not correlate with cause of death.

Liver D1 activity was significantly lower in patients with acute renal failure requiring renal replacement therapy such as dialysis or hemofiltration and in those receiving inotropes compared to those who did not require these treatments (Mann-Whitney U test, P<0.01 for both) (Figs. 3 & 4). Liver D3 activity was higher in patients who had been treated with inotropes (Mann-Whitney U test, P<0.05) or RRT (P=0.056) (Figs. 3 & 4). Muscle D3 activity was also higher in patients who had been treated with inotropes (Mann-Whitney U test, P<0.05) (Fig. 4). Correction for treatment with dopamine did not affect these correlations. Liver D1 activity was negatively correlated with plasma urea (R=-0.35, P=0.01) and with plasma creatinine (R=-0.27, P=0.06) (Fig. 5). A remarkably strong, negative correlation was found for liver D1 activity with plasma total bilirubin (R=-0.54, P<0.0001) (Fig. 6). D3 activities in liver and skeletal muscle showed no relationship with plasma urea, creatinine or bilirubin levels. No correlation was found of liver D1, liver D3 or skeletal muscle D3 activity with plasma C-reactive protein (CRP) levels.

Affinity-labeling of the 27 kDa D1 protein in liver homogenates using BrAc[$^{125}$I]T3 showed a good correlation with the D1 activities determined in the same samples (Fig 7). Affinity-labeling of D1 but not of other proteins was blocked

by the addition of PTU and unlabeled rT3 (not shown), in support of the specificity of the affinity-labeling of D1 with BrAc[$^{125}$I]T3. Liver D1 and D3 mRNA levels determined by real-time quantitative RT-PCR were significantly correlated with the corresponding deiodinase activities (R=0.74, P<0.001, for D1; R=0.78, P< 0.001 for D3) (Fig. 8).

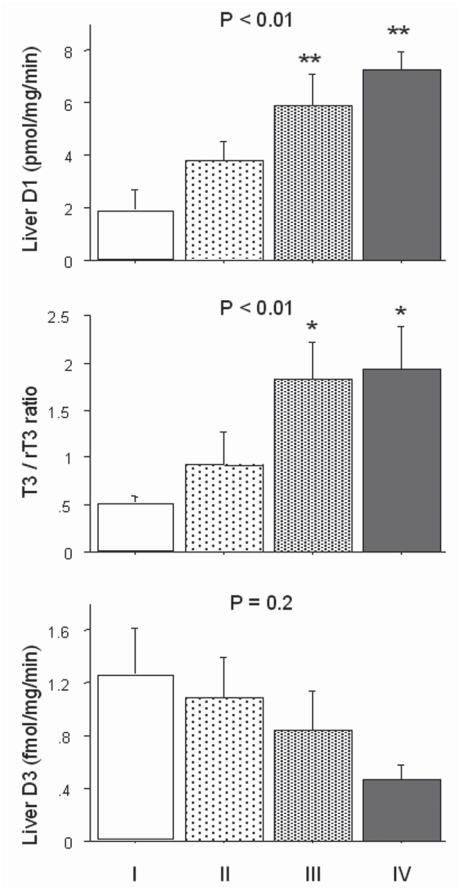



**Figure 2.** Correlation of liver D1 (A) and liver D3 (B) activities, and the T3/rT3 ratio (C) with cause of death in patients who were not treated with thyroid hormone. Patients are divided in four different groups based on cause of death. I = Cardiovascular collapse (n=5), II = MOF sepsis (n=21), III = MOF SIRS (n=14), IV = Severe brain damage (n=4). Liver D1 activity and serum T3/rT3 ratio showed a significant relation with cause of death (P<0.01), whereas liver D3 activity showed an opposite trend.

** P<0.01 versus group I; * P<0.05 versus group I. Data represent means ± SEM and P-values were obtained with ANOVA and Fischer's LSD for multiple comparisons.

**Fig. 3**

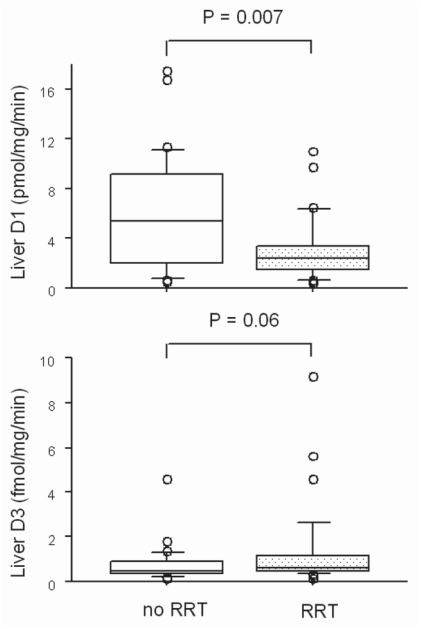



**Figure 3.** Liver D1 (A) and liver D3 (B) activities in patients with acute renal failure requiring renal replacement therapy (RRT) (n=33) and patients who did not require this treatment (n=32). Liver D1 was significantly lower in patients who required RRT (P<0.01), whereas liver D3 activity was higher (P=0.06). Box-plots represent $10^{th}$-$25^{th}$-$50^{th}$-$75^{th}$-$90^{th}$ percentile and P-values were obtained with Mann Whitney-U test.

**Fig. 4**

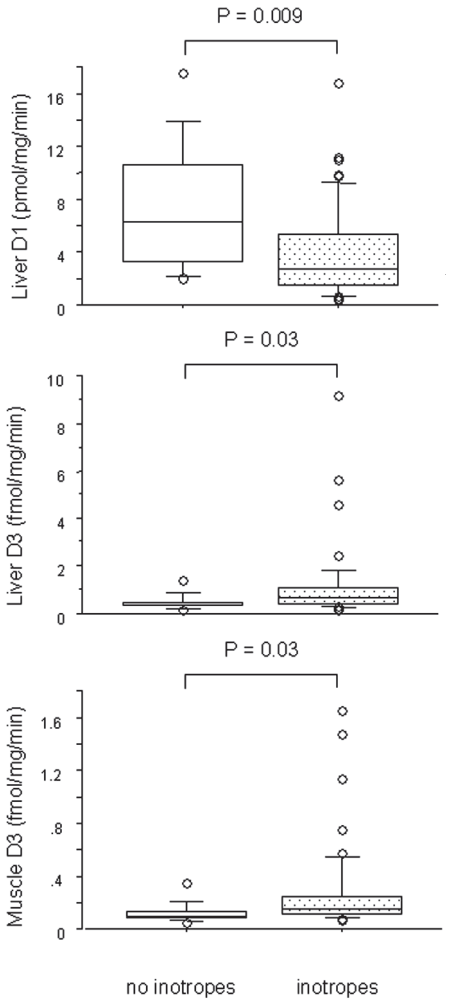
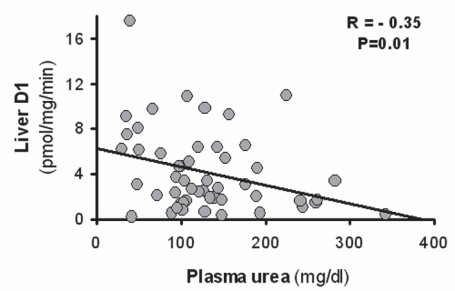



**Figure 4.** Liver D1 (A) and liver D3 (B) activities in patients receiving inotropes (n=54) and patients who did not require this treatment (n=11). Liver D1 was significantly lower in patients who required inotropes (P<0.01), whereas liver D3 activity was higher (P<0.01). Skeletal muscle D3 activity (C) was also higher (P<0.05) in patients who were treated with inotropes (n=53) than in patients who were not (n=12). Box-plots represent $10^{th}$-$25^{th}$-$50^{th}$-$75^{th}$-$90^{th}$ percentile and P-values were obtained with Mann Whitney-U test.



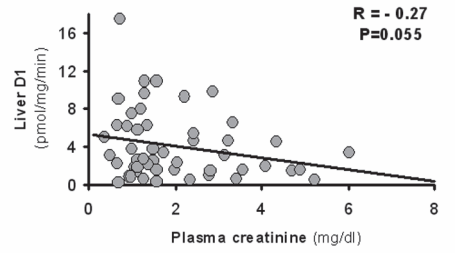




**Figure 5.** Correlation of liver D1 activity with plasma creatinine and plamsa urea levels in 65 patients. Liver D1 activity showed a negative trend with plasma creatinine (P=0.06) and a negative correlation with plasma urea levels (P=0.01).

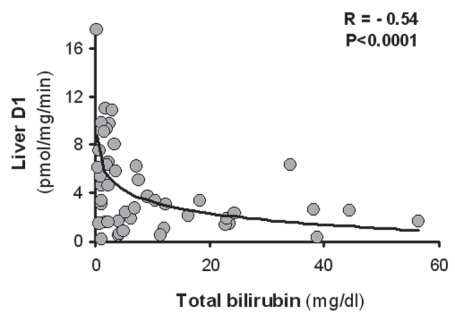



**Figure 6.** Correlation of liver D1 activity with serum bilirubin levels in 65 patients. Liver D1 activity showed a significant negative correlation with serum bilirubin levels (P<0.0001).

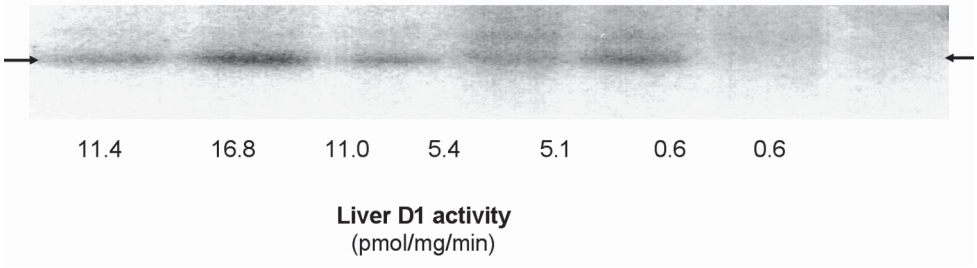



**Figure 7.** Affinity labelling of the 27 kDa protein in liver homogenates using BrAc[$^{125}$I]T3. Liver D1 activity is shown on the bottom of the figure and shows a good corrrelation with the affinity labelling of the 27 kDa D1 protein (arrow).

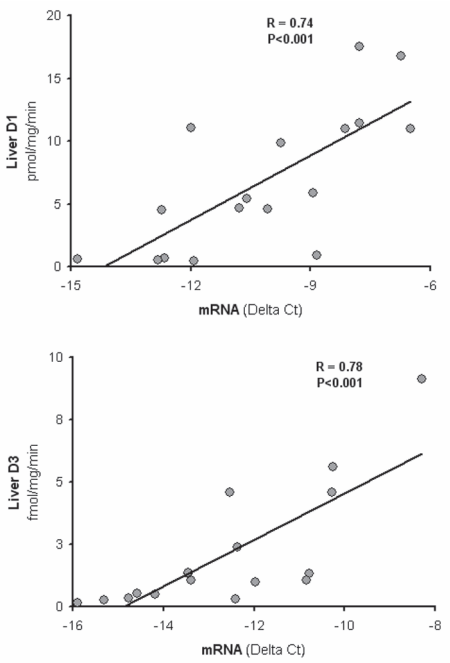



**Figure 8.** Correlation of liver D1 (A) and liver D3 (B) activities with D1 and D3 mRNA levels in a selection of samples with low, intermediate or high activity. mRNA levels were determined by real-time RT-PCR and are expressed as delta Ct, i.e. the Ct value of the target gene minus the Ct value of the housekeeping gene. Liver D1 and D3 activities showed positive correlations with corresponding mRNA levels ($P<0.001$).

## Discussion

Critical illness is associated with reduced TSH and thyroid hormone secretion as well as with marked changes in peripheral thyroid hormone metabolism, resulting in low circulating T3 and high rT3 levels (10). Both the fall in serum T3 and the rise in rT3 have been found to correlate with severity of illness (10) and decreased serum T4 has been associated with poor prognosis. Inactivation of thyroid hormone is presumed to reflect an adaptive response of the body to conserve tissue function. As expected, we found low serum levels of TSH, total T4 and T3, high levels of rT3 and a low active over inactive thyroid hormone (T3/rT3) ratio in the studied critically ill patients. Liver D1 activity was downregulated and liver and skeletal muscle D3 activity, not present in healthy individuals, was induced. Changes in tissue deiodinase activities, particularly pronounced in conditions characterized by low tissue perfusion, were statistically correlated with the altered circulating thyroid hormone levels, suggesting a role in the pathophysiology of the "low T3 syndrome" of severe illness. Previous studies have suggested large changes in serum thyroid hormone levels after death (26). However, correlations observed in our study cannot be explained by differences in post-mortem time, since no relation of post-mortem time with deiodinase activities or serum thyroid parameters was observed.

In healthy subjects, about 20% of circulating T3 is secreted by the thyroid. Estimates of the contribution of liver and kidney D1 to peripheral T3 production vary from 15 to 80% (12), with the remainder of extrathyroidal T3 production originating in D2-containing tissues such as skeletal muscle. The contribution of D1 seems to be highest in hyperthyroid patients, whereas D2 may play a more important role in euthyroid and, in particular, hypothyroid subjects (12). A low D1 activity will not only result in a decreased production of T3 from T4, but also in reduced clearance of rT3, since D1 is the principal pathway for rT3 clearance (12). A decreased uptake of T4 and rT3 into D1-expressing tissues is another possible mechanism to lower serum T3 and increase serum rT3 levels (10, 15, 16). Furthermore, the activity of the different deiodinases greatly depends on thiol cofactors, although the physiological cofactor(s) for each deiodinase has not been identified (11, 12). In our study, tissue deiodinase activities were determined in the presence of excess exogenous cofactor (DTT). Our studies, therefore, do not provide information about changes in the availability of the natural cofactors as a possible mechanism for the changes in peripheral thyroid hormone metabolism in critical illness.

In normal healthy subjects, only D1 is expressed in liver and preliminary data suggest that D2 activity is expressed in skeletal muscle (12). In liver of critically ill patients, we found D1 activity which, except for patients who died acutely from severe brain damage, was low compared with values observed in healthy individuals, and also substantial D3 activity. As expected, D2 activity could not be detected in liver. Surprisingly, skeletal muscle of critically ill patients showed a significant D3 activity, whereas both muscle D1 and D2 activities were negligible. In general, the expression of D1 and D3 is up-regulated and that of D2 is down-regulated by thyroid hormone (11, 12). Based on the low serum thyroid hormone levels in our patients, a high D2 expression and a low D1 and D3 expression would be expected. Although we did observe mostly low hepatic D1 expression, D2 activity in skeletal muscle was undetectable, and liver and skeletal muscle samples expressed substantial D3 activities. Hence, a role of regulators other than thyroid hormone is suggested.

To reduce the confounding effect of variable concentrations of T4 and T4-binding proteins, we mainly focused on the correlation of tissue deiodinase activities with serum iodothyronine ratios. Since a low D1 expression conceivably reduces T3 production and rT3 clearance, and since elevated D3 expression enhances T3 clearance and rT3 production, the serum T3/rT3 ratio is the parameter which most accurately reflects the result of altered peripheral thyroid hormone metabolism during critical illness. Our data suggest an important role of liver D1 in inducing the altered thyroid hormone levels of critically ill patients, since liver D1 activity was

positively correlated with serum T3/rT3 ratio and negatively correlated with the serum rT3/T4 ratio. Surprisingly, liver D1 activity also showed a negative correlation with the serum T3/T4 ratio. This is in part due to the positive correlation between liver D1 activity and serum T4 levels in the thyroid hormone-treated group, which may be explained by the positive control of D1 expression by thyroid hormone, although this is thought to be mediated by T3 (12, 27). Liver D3 activity correlated positively with serum rT3/T4 and tended to correlate negatively with serum T3/rT3. Normally, D3 is only present in human liver during fetal development, where it protects the fetus from undue exposure to thyroid hormone, suggesting that pathological conditions in adult life may be associated with changes in deiodinase expression, in particular that of D3, to levels occurring during fetal development (24, 28). There are two ways by which D3 decreases T3 availability: it prevents conversion of T4 to T3 by catalyzing the conversion of T4 to rT3 instead, and it also catalyzes the degradation of T3 to 3,3'-T2. Therefore, induction of liver D3 in critically ill patients is likely to contribute to the low serum T3 and high serum rT3 levels in critically ill patients.

There is evidence that D2 is expressed in skeletal muscle of healthy subjects (25). D2 activity is regulated by substrate-induced enzyme inactivation (12). Hence, it may be surprising that we failed to detect significant D2 activity in skeletal muscle of critically ill patients, particularly in view of the low serum T4 levels. However, critically ill patients have strongly increased serum rT3 concentrations, which may also inactivate D2 (29). Another explanation for the lack of D2 activity is the short half-life of functional D2 protein (<1 h in euthyroid conditions) which may cause rapid post-mortem D2 inactivation (12). However, since in this study, tissue biopsies were taken within minutes after death, post-mortem decay of D2 activity should be minimal. The shortest interval between entry in the ICU and isolation of tissue samples was between 24 and 48 h. Thus, in view of the short half-life of the D2 protein, it may well have disappeared from skeletal muscle if its expression is acutely suppressed in severe illness. It is also possible that D2 expression varies between different types of skeletal muscle, but this remains to be investigated. Like D1, D2 is a thyroid hormone-activating enzyme. Therefore, the lack of D2 activity in skeletal muscle, as does the down-regulation of hepatic D1 activity, may contribute to low serum T3 levels in critically ill patients.

Another surprising finding was the expression of substantial D3 activity in skeletal muscle. Muscle D3 activity was significantly correlated with serum rT3/T4 ratio, but not with T3/rT3 ratio, in critically ill patients. Although D3 activity has been reported previously in fetal rat muscle (30), this is the first report of D3 expres-

sion in human skeletal muscle. By converting T4 to rT3 and T3 to 3,3'-T2, D3 may lower local thyroid hormone levels in skeletal muscle. It has been shown recently that expression of D3 in hemangiomas may result in very low serum T4 and T3 and very high rT3 levels (17). Since skeletal muscle is such an abundant tissue in humans, it is likely that induction of D3 in skeletal muscle also contributes to the low serum T4 and T3, and high rT3 levels in critical illness.

No significant differences in tissue deiodinase expression were observed between patients who had been treated with intensive or conventional insulin therapy. This seems to contradict the marked beneficial effects of intensive insulin therapy on morbidity and mortality in intensive care patients (8). However, it should be noted that the current study involved post-mortem samples. The illness of patients who died after being intensively treated with insulin was therefore at least as severe as that of patients who died after having received conventional insulin therapy.

Liver D1 activity and serum T3/rT3 ratio showed a significant relationship with cause of death. Cause of death was categorized in four different groups, with group 1 having the most severe, and group 4 the least hemodynamic instability prior to death. Hepatic D1 activity and serum T3/rT3 were lowest in patients who died of a cardiovascular collapse (group 1), and were highest in patients who died of severe brain damage (group 4). Liver D3 showed an opposite, insignificant correlation with cause of death. Since ICU stay was shorter in groups 1 and 4 than in groups 2 and 3 (MOF with sepsis and MOF with SIRS), these correlations cannot be explained by the duration of the illness. These data suggest that the observed changes in liver deiodinase activity may relate to poor tissue perfusion. For this reason, we analyzed the relation of tissue deiodinase activities with several other clinical parameters related to a decreased tissue perfusion, such as decreased kidney function (plasma urea and creatinine), the need for renal replacement therapy (dialysis or hemofiltration), the need for inotropes, and impaired liver function (plasma total bilirubin). Liver D1 activity was negatively correlated with plasma urea and creatinine. Patients with acute renal failure requiring dialysis/hemofiltration and those requiring inotropes for hemodynamic stability had lower liver D1 activities and higher liver D3 activities than patients who did not require these treatments. Skeletal muscle D3 activity was also higher in patients treated with inotropes. Although dopamine is known to inhibit TSH secretion and peripheral T4 to T3 conversion (31), correction for dopamine did not affect these correlations. Liver D1 activity showed a very strong negative correlation with plasma total bilirubin, whereas no correlation was shown with inflammation as reflected by an elevated CRP level. To our knowledge, there are no previous data suggesting a relation between deiodinase activity and tis-

sue hypoxia, but a pH dependence of D1 activity has been shown in perfused rat livers (32). Since D1 is responsible for the activation and D3 for the inactivation of thyroid hormone, regulation of deiodinase activities by cellular hypoxia may be a tissue-specific way to alter thyroid hormone bioactivity during limited oxygen supply.

Our data regarding hepatic deiodinase activities were substantiated by a good correlation with liver D1 and D3 mRNA levels and by affinity-labeling of the D1 protein with N-bromoacetyl-[$^{125}$I]T3. This suggests that the changes in liver D1 and D3 expression in severely ill patients are largely exerted at the pretranslational level.

In conclusion, this is the first report on the relationships between tissue deiodinase activities, thyroid hormone levels and clinical parameters in a large group of critically ill patients. Liver D1 activity is down-regulated, and liver and skeletal muscle D3, both not present in healthy subjects, are induced, particularly in those disease states with poor tissue perfusion and unrelated to inflammation. The data suggest that low D1 activity plays an important role in the altered thyroid hormone levels during critical illness. The induction of D3 in liver and skeletal muscle of critically ill patients suggests a role of this enzyme in pathophysiology, which may be more important than previously thought. Indeed, high D3 activity in an abundant tissue such as skeletal muscle is likely to lower circulating T4 and T3 and increase rT3 levels, hence contributing to the "low T3 syndrome" of severe illness.

## Acknowledgements

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# *Chapter B2*

## Serum rT3 and T3/rT3 are prognostic markers in critically ill patients and are associated with post-mortem tissue deiodinase activities

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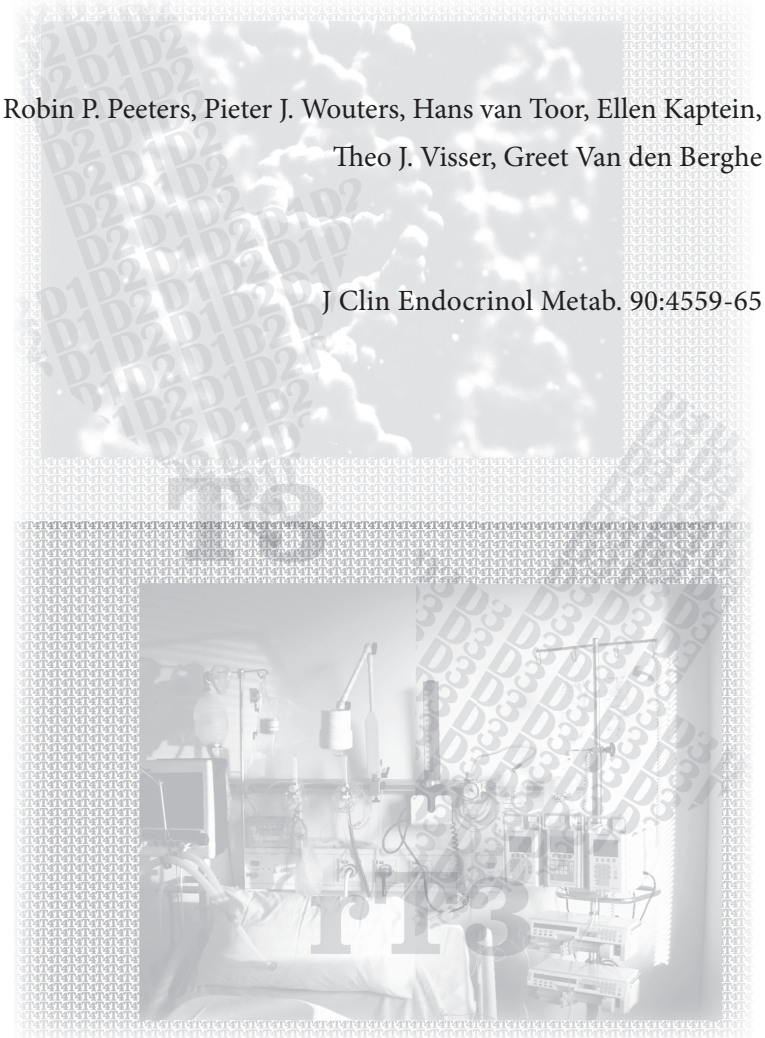

## Abstract

**Introduction and methods**: Critical illness is associated with reduced TSH and thyroid hormone secretion, and with changes in peripheral thyroid hormone metabolism, resulting in low serum T3 and high rT3. In 451 critically ill patients who received intensive care for more than 5 days, serum thyroid parameters were determined on day 1 (d1), d5, d15 and last day (LD). All patients had been randomized for intensive or conventional insulin treatment. 71 patients died, and post-mortem liver and skeletal muscle biopsies were obtained from 50 of them for analysis of deiodinase (D1-3) activities.

**Results:** Insulin treatment did not affect thyroid parameters. On d1, rT3 was higher and T3/rT3 lower in non-survivors as compared with survivors (P=0.001). Odds ratio for survival of the highest vs the lowest quartile was 0.3 for rT3 and 2.9 for T3/rT3. TSH, T4, and T3 were lower in non-survivors from d5 until LD (P<0.001). TSH, T4, T3 and T3/rT3 increased over time in survivors, but decreased or remained unaltered in non-survivors. Liver D1 activity was positively correlated with LD serum T3/rT3 (R=0.83, P<0.001), and negatively with rT3 (R=-0.69, P<0.001). Both liver and skeletal muscle D3 activity were positively correlated with LD serum rT3 (R=0.32, P=0.02 and R=0.31, P=0.03).

**Conclusion:** In critically ill patients who required more than five days intensive care, rT3 and T3/rT3 were already prognostic for survival on d1. On d5, T4, T3, but also TSH levels are higher in patients who will survive. Serum rT3 and T3/rT3 were correlated with post-mortem tissue deiodinase activities.

## Introduction

During critical illness, pronounced alterations in the hypothalamic-pituitary-thyroid axis occur without any evidence for thyroid disease (1, 2). The alterations during this "non-thyroidal illness" (NTI) are different between the acute and the chronic phase (3, 4). Within a few hours after onset of acute stress, plasma T3 decreases and plasma rT3 increases. The magnitude of these changes is related to the severity of the disease (2, 5-7). In severely ill patients, also T4 decreases and both T4 and T3 are inversely correlated with mortality rate (2, 8). Despite a major decrease in T3, TSH levels remain within the normal range. The nocturnal TSH surge that occurs in the physiologic state, is already absent in the acute stage of critical illness (9-11). In prolonged critical illness, patients have even lower levels of circulating T3, a decreased T4, and a low to normal plasma TSH with a dramatic reduction in the pulsatile TSH secretion (3, 4, 12).

The normal or low plasma TSH levels in critically ill patients, despite decreased T4 and T3 levels, suggest a major change in setpoint within the hypothalamus-pituitary-thyroid (HPT) axis. Indeed, a post-mortem study of patients with prolonged illness showed a decreased TRH mRNA expression in the hypothalamic paraventricular nucleus, that was correlated with decreased serum TSH and T3 (13). The combination of decreased serum T3 and increased serum rT3 levels suggests that also major changes in the peripheral metabolism of thyroid hormone occur. Reduced activation by type I deiodinase (D1) and increased inactivation by D3 have been shown in post-mortem liver and skeletal muscle tissues of NTI patients (1, 14). An altered transport of thyroid hormone into D1 and D3 expressing tissues may also play a role (15).

Whether the reduction in serum T3 is a beneficial adaptation resulting in a decreased metabolic rate and a protection against hypercatabolism or whether it is a mal-adaptation contributing to a worsening of the disease is still a controversial issue (2, 3, 16). So far, it has not been clearly demonstrated that substitution of critically ill patients with thyroid hormone has a positive effect on clinical outcome (17-19). Intervention with hypothalamic releasing factors, which restores pulsatile pituitary hormone secretion, normalizes peripheral hormone levels, and keeps the negative feedback loop intact, might be a more successful approach (20-22).

In a recent study involving 1548 intensive care unit (ICU) patients, it was shown that maintaining normoglycemia during critical illness, using intensive insulin treatment, reduced ICU mortality by 43% and hospital mortality by 34% (23). Furthermore, morbidity was reduced resulting in a decreased need for prolonged mechanical ventilation, dialysis, red blood cell transfusion, antibiotic therapy and intensive care. Previously it was shown that control of hyperglycemia in patients with either type I or type II diabetes is associated with normalization of thyroid hormone concentrations (24-26). The metabolic effects and / or the clinical benefits of insulin may therefore affect thyroid hormone secretion and metabolism during critical illness.

In this study we investigated the effect of intensive insulin therapy on serum thyroid hormone levels in prolonged critically ill patients who received intensive care therapy for at least 5 days. Furthermore, we analysed deiodinase activities in tissues of the patients who died in the ICU, and investigated correlation with serum thyroid hormone levels during intensive care.

## Patients and Methods

### *Patients*

This study was part of a large randomized controlled study on intensive insulin therapy in ICU patients (N=1548), of which the major clinical outcomes have been published in detail elsewhere (23). All mechanically ventilated adult patients were eligible for inclusion in this trial after informed consent from the closest family member. On admission, patients were randomly assigned to either strict normalization of blood glucose (80-110 mg/dl) with intensive insulin therapy or the conventional approach, in which insulin infusion is initiated only when blood glucose exceeds 215 mg/dl and which resulted in an average blood glucose of 150-160 mg/dl. The study protocol had been approved by the Ethical Review Board of the Catholic University of Leuven School of Medicine.

**Table 1.** Descriptive statistics of the population used in this study

| | Conventional Insulin treatment | Intensive Insulin treatment | ***P*** |
|---|---|---|---|
| No. of patients | 243 | 208 | |
| Age (years) | 61.3 ± 15.7 | 61.6 ± 15.1 | 0.97 |
| No. of male patients (%) | 164 (67.5 %) | 144 (69.2 %) | 0.69 |
| BMI (kg/m$^2$) | 25.6 ± 5.6 | 25.7 ± 4.6 | 0.36 |
| ICU stay (days) | 15 (9-27) | 12 (8-21) | **0.003** |
| APACHE II score Day 1 | 12 (8-15) | 11 (7-15) | 0.70 |
| TISS-28 score Day 1 | 39 (33-45) | 40 (35-45) | 0.50 |
| No. of patients receiving TH treatment (%) | 38 (15.6 %) | 24 (11.5 %) | 0.21 |
| No. of patients receiving TH treatment on d 1 (%) | 2 (0.8 %) | 3 (1.4 %) | 0.53 |
| No. of patients receiving TH treatment on d 5 (%) | 18 (7.4 %) | 8 (3.8 %) | 0.11 |
| No. of patients receiving TH treatment on d 15 (%) | 15 (8.9 %)[a] | 12 (13.8 %)[b] | 0.86 |
| No. of patients receiving TH treatment on Last d (%) | 32 (13.2 %) | 22 (10.6 %) | 0.40 |
| No. of patients receiving GC treatment (%) | 79 (32.5%) | 70 (33.7%) | 0.80 |
| No. of patients receiving Dopamine treatment (%) | 56 (23%) | 56 (26.9%) | 0.38 |

[a] N=123 , [b] N= 87
Age and BMI are shown as mean ± SD, ICU stay, APACHE II and
TISS-28 scores are shown as median (IQR). P values represent Mann-Whitney U tests.
TH, Thyroid Hormone; GC, Glucocorticoids; IQR, Interquartile range

For the current analysis, all patients with an ICU stay of more than 5 days were included (n=451). Table 1 describes the baseline characteristics of the two treatment groups. Blood samples were obtained at 0600h on day 1, day 5, day 15, and on the last day of intensive care. 71 patients died in the ICU, and liver and skeletal muscle (rectus abdominis) biopsies were available from 50 patients. Biopsies were taken within minutes after death. 62 patients had been treated with thyroid hormone at some point during the course of their critical illness. Treatment was initiated when they had a serum T4 concentration below 50 nmol/l in the face of a normal TBG level and concomitantly clinical symptoms of hypothyroidism, defined as coma or central nervous system suppression, failure to wean from the ventilator, or hemodynamic instability, which were unexplained and resistant to conventional supportive therapy. In these cases, thyroid hormone treatment consisted of an IV bolus of 150 μg T4 daily plus 0.6 μg T3 per kg body weight per 24 h as a continuous IV infusion.

### *Serum analyses*

The care of patients in the ICU often comprises infusion of heparin, either systemically or locally to prevent clotting of vascular access, which substantially interferes with the assay used to quantify free concentrations of thyroid hormone (27). Therefore, we refrained from measuring serum FT4 and FT3 in this study. Serum total T4, total T3, and TSH were measured by chemoluminescence assays (Vitros ECi Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK). rT3 was measured by radioimmunoassay as previously described (28). Within assay coefficients of variation amounted to 4% for TSH, 2% for T4, 2% for T3, and 3-4% for rT3. Normal values for TSH, T4, T3, and rT3 were determined in 270 healthy individuals. Mean ± 2 SD was used as the normal range for T4, T3, and rT3, whereas the 95% confidence interval was used for TSH.

### *Deiodinase activities*

Human liver and skeletal muscle samples were homogenized on ice in 10 volumes of PE buffer (0.1 M phosphate, 2 mM EDTA, pH 7.2) using a Polytron (Kinematica AG, Lucerne, Switzerland). Homogenates were snap-frozen in aliquots and stored at -80 C until further analysis. Protein concentration was measured with the Bio-Rad Protein Assay using BSA as the standard following the manufacturer's instructions.

Liver D1 activities were determined as described earlier (14) by duplicate incubations of homogenates (10 μg protein) for 30 min at 37 C with 0.1 μM [3',5'-$^{125}$I]rT3 (100,000 cpm) in a final volume of 0.1 ml PED10 buffer (PE + 10 mM DTT). Skeletal muscle D2 activities were assayed as earlier described (14) by du-

plicate incubation of 200 µg of homogenate protein for 60 min at 37 C with 1 nM [3',5'-$^{125}$I]T4 (100,000 cpm) in a final volume of 0.1 ml PED25 buffer (PE + 25 mM DTT). The incubations were carried out in the presence of 0.1 µM unlabeled T3, to prevent inner ring deiodination of the labeled T4 substrate by D3, if present, and in the absence or presence of 0.1 µM unlabeled T4 which is sufficient to saturate D2. Deiodination of labeled T4 in the absence minus that in the presence of excess unlabeled T4 represents D2 activity. The further procedure for the quantitation of $^{125}I^-$ production was the same as described above for the D1 assay. Tissue D3 activities were measured as described earlier (14) by duplicate incubation of liver (100 µg protein) or skeletal muscle (200 µg protein) homogenate for 60 min at 37 C with 1 nM [3'-$^{125}$I]T3 (200,000 cpm) in a final volume of 0.1 ml PED50 buffer (PE + 50 mM DTT).

### *Statistical analysis*

Data were analyzed using the statistical program SPSS 10.0.7 for Windows (SPSS, Chicago, IL). To minimize the influence of outliers, all data were analyzed using non-parametric tests. Patients who had been treated with thyroid hormone were excluded from all analyses shown, except for the correlation of serum levels with tissue deiodinase activities. Data are shown as mean ± SD or as median (IQR), depending on the distribution. Differences between the groups were analyzed using Mann-Whitney U tests, correlation coefficients represent Spearmann's correlation coefficient. Statistical significance was assumed for $P < 0.05$.

## Results

### *Intensive insulin treatment does not affect serum thyroid parameters*

The baseline characteristics of the two treatment groups are shown in Table 1. There was no difference in age, gender, severity of disease, or need for thyroid hormone treatment between the two treatment groups, but patients in the intensive insulin treatment group stayed significantly shorter in the ICU. At none of the time points (d1, d5, d15, and LD) were serum TSH and iodothyronines different between the conventional and intensive insulin treatment group, although TSH and T4 tended to be lower in the intensively treated group on the last day (P=0.08 and P=0.06, respectively). It is most likely that only patients who were treated with thyroid hormone prior to their hospitalization, received thyroid hormone substitution from day 1. However, thyroid hormone substitution before admission was not documented of all patients.

**Table 2.** The difference in serum TSH and iodothyronine levels between survivors and non-survivors.

| | | Survivor | Non-survivor | P |
|---|---|---|---|---|
| TSH | Day 1 | 0.50 [0.16-1.17] (353) | 0.39 [0.13-1.28] (68) | 0.46 |
| (μU/ml) | Day 5 | 1.22 [0.49-2.27] (346) | 0.42 [0.12-1.32] (62) | **<0.001** |
| | Day 15 | 1.45 [0.67-2.44] (144) | 0.84 [0.25-2.38] (28) | **0.05** |
| | Last Day | 1.49 [0.77-2.33] (332) | 0.44 [0.04-0.95] (38) | **<0.001** |
| T4 | Day 1 | 4.62 [3.65-5.87] (366) | 4.37 [3.43-5.74] (69) | 0.19 |
| (μg/dL) | Day 5 | 5.66 [4.09-7.20] (356) | 3.35 [2.46-5.29] (63) | **<0.001** |
| | Day 15 | 6.72 [4.92-8.24] (148) | 3.91 [2.89-6.92] (29) | **<0.001** |
| | Last Day | 7.49 [6.10-8.78] (344) | 3.40 [2.00-5.44] (39) | **<0.001** |
| T3 | Day 1 | 63.0 [48.7-79.2] (365) | 57.8 [47.4-75.9] (69) | 0.32 |
| (ng/dL) | Day 5 | 74.0 [59.1-92.2] (356) | 53.9 [42.2-70.7] (63) | **<0.001** |
| | Day 15 | 87.0 [66.2-107.7] (148) | 61.0 [50.6-77.2] (29) | **<0.001** |
| | Last Day | 94.1 [77.9-109.7] (344) | 54.5 [42.2-70.1] (39) | **<0.001** |
| rT3 | Day 1 | 38.3 [26.0-57.1] (363) | 55.2 [31.2-91.5] (69) | **<0.001** |
| (ng/dL) | Day 5 | 40.9 [27.9-65.6] (356) | 59.1 [35.0-97.4] (63) | **<0.001** |
| | Day 15 | 33.7 [23.4-63.6] (149) | 63.6 [31.8-101.2] (28) | **0.001** |
| | Last Day | 37.6 [26.0-54.52 (343) | 90.9 [40.2-135.0] (39) | **<0.001** |
| T3 / rT3 | Day 1 | 1.69 [0.98-2.49] (362) | 1.19 [0.62-2.15] (69) | **0.001** |
| (Molar ratio) | Day 5 | 1.78 [1.07-3.05] (354) | 1.00 [0.54-1.50] (63) | **<0.001** |
| | Day 15 | 2.62 [1.30-3.87] (148) | 0.95 [0.56-2.18] (28) | **<0.001** |
| | Last Day | 2.54 [1.57-3.82] (342) | 0.61 [0.39-1.23] (39) | **<0.001** |
| T3 / $T_4$ | Day 1 | 0.016 [0.013-0.019] (365) | 0.017 [0.013-0.021] (69) | 0.39 |
| (Molar ratio) | Day 5 | 0.017 [0.014-0.020] (356) | 0.019 [0.016-0.024] (63) | **0.004** |
| | Day 15 | 0.016 [0.014-0.019] (148) | 0.019 [0.013-0.022] (29) | **0.05** |
| | Last Day | 0.015 [0.013-0.018] (344) | 0.020 [0.013-0.028] (39) | **<0.001** |

Data are shown as median [IQR]. the number of patients in each group is shown between brackets. P values represent Mann-Whitney U tests. IQR, Interquartile range
Reference values in our lab are 0.4 – 4.3 μU/ml for TSH, 4.51 – 9.95 μg/dL for $T_4$, 92.8 – 162.9 ng/dL for $T_3$, and 9.1 – 22.1 ng/dL for $rT_3$.

### *Serum TSH and iodothyronines are different between survivors and non-survivors*

Table 2 shows serum TSH and iodothyronine levels of the survivors and non-survivors in this study. Reference values in our lab are 0.4 – 4.3 μU/ml for TSH, 4.51 – 9.95 μg/dL for T4, 92.8 – 162.9 ng/dL for T3, and 9.1 – 22.1 ng/dL for rT3. From day 5 onward, serum TSH, T4, and T3 were substantially lower in patients who will ultimately die. Serum rT3 and T3/rT3 were already different on day 1, with rT3 being higher and T3/rT3 being lower in non-survivors. Already on day 1, the odds ratio for survival of the highest vs. the lowest quartile was 0.30 for rT3 and 2.9 for T3/rT3. To analyze the predictive value of these parameters, we created Receiver Operating Characteristic (ROC) curves for mortality. These revealed that the predictive value of both rT3 and T3/rT3 on day 1 was low (area under the curve was 0.68 for rT3 and 0.65 for T3/rT3), due to the overlap between survivors and non-survivors, and somewhat better on the last day (area under the ROC curve (AUROC) was 0.76 for rT3 and 0.84 for T3/rT3). The AUROC of T3/rT3 on the last day is comparable to that of APACHE II and of IGFBP-1 (29). Figure 1 shows the ROC curve of the T3/rT3 ratio on both day 1 and the last day.

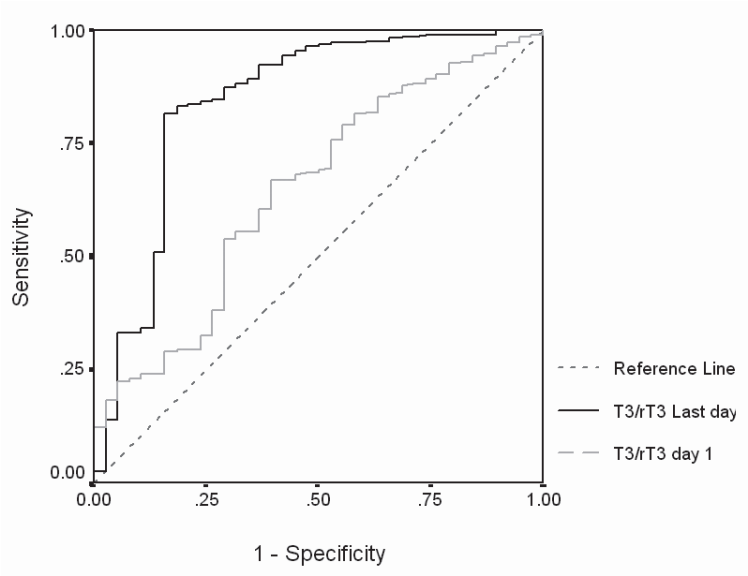


**Figure 1.** A Receiver Operating Characteristic (ROC) curve of the T3/rT3 ratio on both day 1 and the last day. The area under the curve on day 1 for T3/rT3 was 0.65 on day 1 and 0.84 on the last day. Diagonal segments are produced by ties.

Since intensive insulin therapy has shown to affect outcome in critically ill patients (23), the odds ratio on day 1 was also calculated for the two insulin-treated groups separately. The odds ratio for survival on day 1 was 0.27 for rT3 and 2.9 for T3/rT3 in the patients treated with conventional insulin therapy, and 0.36 and 2.7, respectively, in the patients treated with intensive insulin therapy. On the last day, the odds ratio for survival was 0.14 for rT3 and 25.3 for T3/rT3 for the whole group.

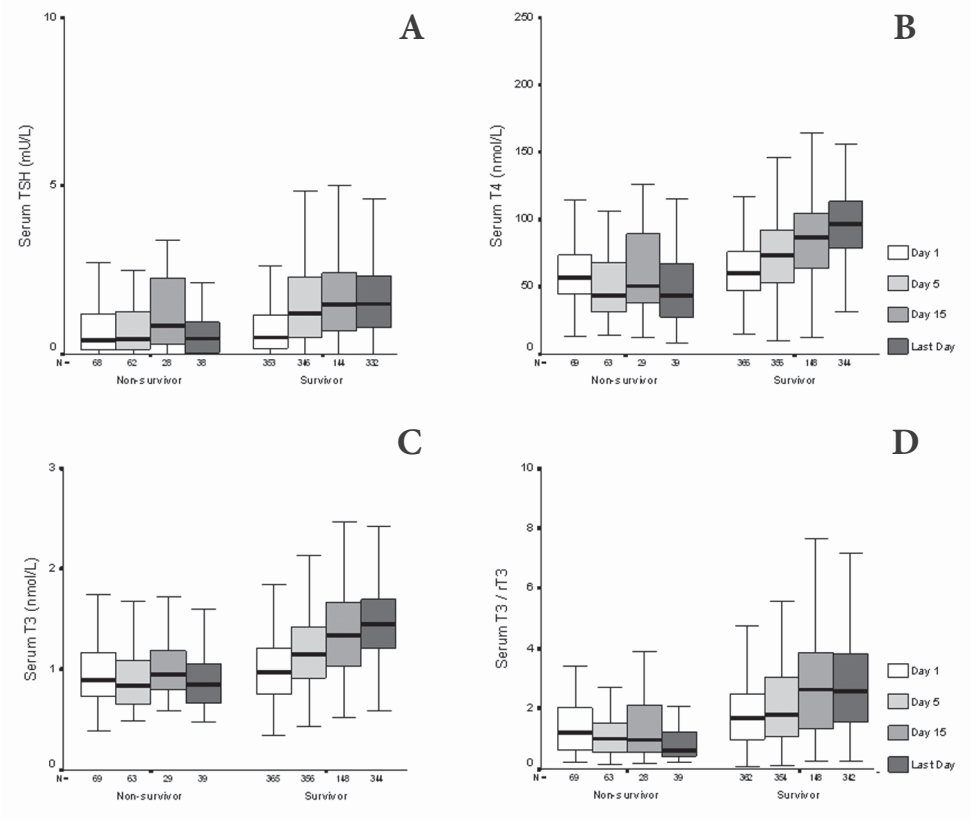


**Figure 2.** Serum TSH, T4, and T3 levels and the T3/rT3 ratio at d1, d5, d15, and LD of ICU stay between survivors and non-survivors. From day 5 onward, serum TSH, T4, and T3 increased in patients who survived, whereas there was no such pattern in patients who died (**A-C).** The serum T3/rT3 ratio increased in survivors from day 5 to LD, whereas it did not alter or even decreased in non-survivors **(D)**. On the last day of ICU stay, the majority of patients had TSH and T4 levels within the normal range, whereas T3 was still low. Patients who had been treated with thyroid hormone were excluded from these analyses. Exclusion of patients who received glucocorticoids or dopamine gave similar results for all serum parameters. Box plots represent 10th-25th-50th-75th-90th percentiles. To convert values for T4 to μg/dL, multiply by 0.0777, and to convert values for T3 or rT3 to ng/dL, multiply by 64.9.

After day 1, serum TSH, T4, and T3 increased in patients who survived, whereas there was no such pattern in patients who died (Fig. 2). There was a further increase in T4 and T3 from day 5 to day 15 and the last day, whereas TSH remained elevated from day 5 onward as compared to day 1. On the last day of intensive care, the majority of survivors had TSH and T4 levels within the normal range, whereas T3 was still decreased. Serum rT3 levels were clearly elevated both in survivors and non-survivors throughout their stay in the ICU. In patients who did not survive, there was a substantial increase in rT3 levels on the day they died. Exclusion of patients who received glucocorticoids or of patients who were treated with dopamine at some point during intensive care gave similar results for all serum parameters.

The serum T3/rT3 ratio increased in survivors from day 5 to LD, whereas it did not change or even decreased in non-survivors (Fig. 2). However, other than for TSH, T4, and T3, this increase in T3/rT3 in survivors occurred only after day 5. A similar course of the T3/rT3 was seen after exclusion of patients who received glucocorticoids or dopamine during intensive care.

### *Correlation of liver D1 and liver and skeletal muscle D3 with serum iodothyronines*

As described previously, we were not able to measure any D2 activity in the skeletal muscle samples of these patients, whereas D3 was induced in both liver and skeletal muscle (14). Post-mortem D1 activity showed a strong, negative correlation with last day serum rT3 ($R=-0.69$, $P<0.001$) and a positive correlation with last day serum T3/rT3 ($R=0.83$, $P<0.001$) (Fig. 3), but not with T4 or T3 (Table 3). Post-mortem liver and muscle D3 activities showed a significant positive correlation with serum rT3 levels (Fig. 3), but were not significantly associated with the T3/rT3 ratio (Table 3). Furthermore, liver but not skeletal muscle D3 was negatively associated with serum T3 levels (Table 3).

**Table 3.** Correlation of tissue deiodinase activities and last day serum thyroid parameters.

| | | Liver D1 | Liver D3 | Muscle D3 |
|---|---|---|---|---|
| TSH | R | 0.22 | -0.21 | -0.32 |
| (μU/ml) | P | 0.12 | 0.14 | **0.03** |
| T4 | R | -0.17 | 0.17 | -0.03 |
| (μg/dL) | P | 0.23 | 0.24 | 0.82 |
| T3 | R | 0.14 | -0.31 | -0.15 |
| (ng/dL) | P | 0.33 | **0.03** | 0.30 |
| rT3 | R | -0.69 | 0.32 | 0.31 |
| (ng/dL) | P | **<0.001** | **0.02** | **0.03** |
| T3 / rT3 | R | 0.83 | -0.20 | -0.27 |
| (Molar ratio) | P | **<0.001** | 0.18 | 0.06 |

R represents Spearman's Correlation coefficient,
N=50 for liver, N=49 for skeletal muscle.

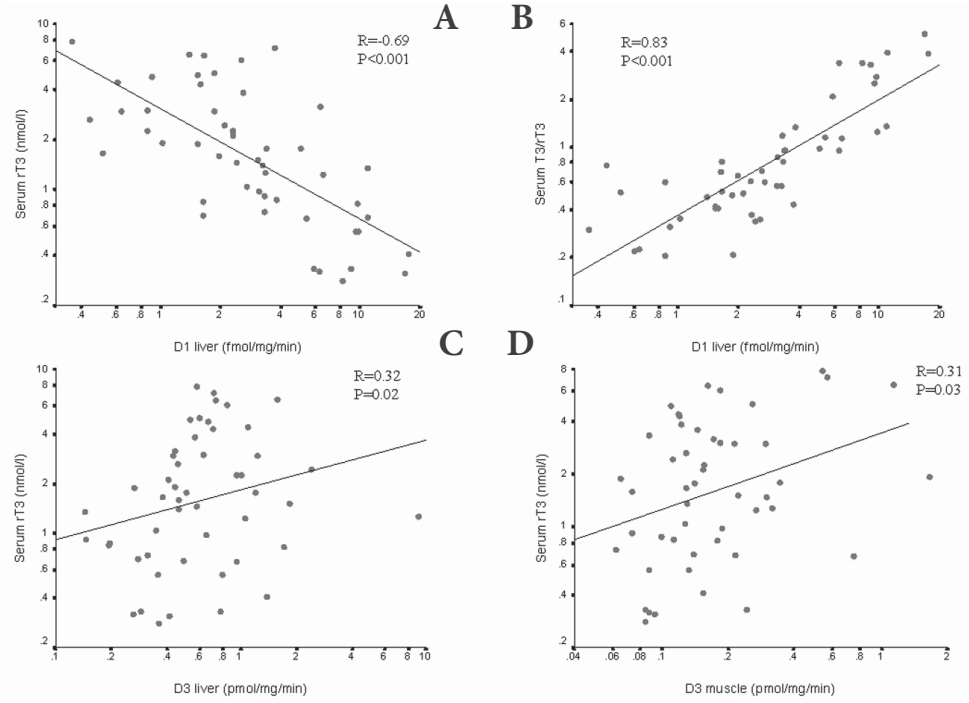


**Figure 3.** Correlation analysis of liver D1 activity and serum rT3 (A) and T3/rT3 (B), and of liver D3 activity (C) and skeletal muscle D3 activity (D) and serum rT3. Liver D1 showed a significant negative correlation with rT3 (P<0.001) and a significant positive correlation with T3/rT3 ratio (P<0.001). Both liver and skeletal muscle D3 activity were positively correlated to serum rT3 levels (P<0.05). No distinction was made in this figure between patients who received thyroid hormone treatment and patients who did not. R represents Spearman's correlation coefficient. To convert values for rT3 to ng/dL, multiply by 64.9.

## Discussion

No difference in thyroid parameters was found between critically ill patients who were treated with intensive versus conventional insulin therapy. In prolonged critically ill patients (in ICU for more than five days), rT3 and the active over inactive thyroid hormone (T3/rT3) ratio on day 1 were already prognostic, albeit weakly, for survival. From day 5 of intensive care onward, TSH, T4, T3 and in a later stage T3/rT3 increased in survivors but decreased or remained unaltered in non-survivors. Serum rT3 and T3/rT3 during ICU stay were correlated with post-mortem tissue deiodinase activity.

During critical illness, plasma T3 decreases and rT3 increases, and the magnitude of these changes is related to the severity of illness (2, 5-7). Intensive in-

sulin therapy has been shown to result in a decreased morbidity and mortality in intensive care patients (23, 30), and it has been shown that control of hyperglycemia in patients with diabetes is associated with normalization of thyroid hormone concentrations (24-26). However, no difference in serum thyroid parameters was observed between patients treated with intensive and conventional insulin therapy, suggesting that neither insulin per se, nor its metabolic effects or its clinical benefits, resulted in altered thyroid hormone levels. For this study, only patients who stayed in the ICU for more than five days were selected. Since intensive insulin therapy also resulted in a shorter period of ICU stay (23), the possibility of a selection bias (sicker patients in the studied group receiving intensive insulin therapy) cannot be excluded. Furthermore, an effect of insulin on the nocturnal TSH peak cannot be excluded from these data, although this is unlikely since such an effect would have resulted in differences in serum T4 and T3 levels (26).

Previous studies have shown that patients with more pronounced alterations in serum thyroid parameters have a significantly higher mortality rate (1, 2, 7, 31, 32). Although all patients required more than five days of intensive care, rT3 and T3/rT3 on day 1 were already prognostic for survival, whereas TSH, T4 and T3 were significantly different between survivors and non-survivors from day 5 onward. Not only the absolute values, but also the time course was completely different between survivors and non-survivors. TSH, T4, and T3 increased in patients who survived, whereas there was no such rise in patients who died. T4 and T3 continued to increase from day 1 to the last day, whereas TSH did not further rise after day 5, suggesting that both T4 and T3 follow the initial increase in TSH. This is in line with previous observations suggesting that an increase in serum TSH drives the rise in T4 and marks the onset of recovery (33, 34). This driving TSH surge is likely to be mediated by TRH. Indeed, hypothalamic TRH gene expression was previously found to be positively correlated with serum TSH and T3 during prolonged critical illness (13), and infusion of TRH in the critically ill could normalize peripheral thyroid hormone levels (20-22). However, a full recovery of the HPT axis in this phase of illness, combined with the low T3 levels, would have required an elevated level of TSH. This was not observed in the current study, whereas it has been described by others (33, 34). This difference cannot be explained by the treatment with glucocorticoids or dopamine, since exclusion of all patients who were treated with these drugs gave similar results. It may be due to the infrequent serum analysis however, since increased levels of TSH were seen in certain, but not all patients.

Serum rT3 levels were clearly elevated in both survivors and non-survivors throughout the intensive care, and rT3 levels did not decrease in survivors. Inter-

estingly, in patients who did not survive, there was a substantial further rise in rT3 levels towards the last day. This might be explained by a short half-life of rT3 (ca 3 hrs vs. ca 24 hrs for T3) (7, 35-37), making rT3 a sensitive marker for acute changes in thyroid hormone metabolism that are caused by peri-mortem tissue decay.

On the last day of intensive care, the majority of patients had TSH and T4 levels back within the normal range, whereas T3 and rT3 remained outside the normal range. Thus, T3 and rT3 levels are not only the first to change in the acute phase of illness (1, 2, 4, 7), but also the last ones to recover.

Serum iodothyronine levels depend not only on the activities of iodothyronine-metabolizing enzymes, but also, among other things, on thyroid function and serum iodothyronine-binding capacity. Because of the confounding effect of variable concentrations of T4 and T4-binding proteins, the serum T3/rT3 ratio is the parameter that most accurately reflects the result of altered peripheral thyroid hormone metabolism during critical illness. The serum T3/rT3 ratio increased with time in survivors, whereas it did not alter or even decreased in non-survivors. This increase in T3/rT3 in survivors occurred only after day 5, suggesting that the peripheral metabolism recovers at a later stage than the centrally initiated TSH secretion.

A low D1 activity will result in a reduced T3 production and rT3 clearance (7). On the other hand, D1 expression is under positive control by thyroid hormone, which is thought to be mediated by T3 (7, 38). In agreement with this, liver D1 was negatively correlated with rT3 and positively with T3/rT3. An induced D3 expression enhances T3 clearance and rT3 production (7, 14), and in line with this, liver and skeletal muscle D3 were positively correlated with rT3, whereas liver D3 also showed a negative correlation with serum T3. Our data suggest an important role of liver D1, and of liver and skeletal muscle D3 in altering thyroid hormone levels in critically ill patients. Especially the very strong correlation between liver D1 and the serum T3/rT3 ratio is remarkable, since serum samples were obtained at 6.00 h on the day the patient died, and deiodinase activities were measured in post-mortem biopsies.

It should be noted that we were not able to detect any D2 in skeletal muscle samples of these patients, whereas D2 activity is present in skeletal muscle of normal subjects (7, 39). There is evidence that D2 in skeletal muscle also contributes to serum T3 production, especially in the hypothyroid situation (7, 39, 40). It is therefore likely that a decreased T4 to T3 conversion by skeletal muscle D2 may also contribute to the low T3 levels in critically ill patients.

In conclusion, in critically ill patients who required intensive care for more than five days, rT3 and T3/rT3 on day 1 were already prognostic for survival al-

though the ROC curve revealed a rather low predictive value. From day 5 onward, TSH, T4, T3 levels, and in a later stage T3/rT3 increased in survivors, suggesting that recovery of thyroid parameters is initiated centrally. The centrally initiated increase in TSH secretion was followed by a recovery of the peripheral metabolism. Serum rT3 and T3/rT3 were significantly correlated with post-mortem deiodinase activity.

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# *Chapter B3*

## Increased T4S levels in critically ill patients due to a decreased hepatic type I deiodinase activity

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## Abstract

**Introduction**: Marked changes in peripheral thyroid hormone metabolism occur in critical illness, resulting in low serum T3 and high rT3 levels. In this study we investigated whether T4S levels are increased in patients who died after intensive care, and if T4S levels are correlated with liver type I deiodinase (D1) or sulfotransferase (SULT) activity.

**Methods:** 64 blood samples and 65 liver biopsies were obtained within minutes after death from 79 intensive care patients, randomized for intensive or conventional insulin treatment. Serum T4S and the activities of hepatic D1 and T2- and E2-SULT were determined.

**Results:** No differences in T4S or hepatic SULT activities were found between patients treated with intensive or with conventional insulin therapy. T4S levels were significantly elevated compared to healthy references. Furthermore, hepatic D1, but not SULT activity, showed a strong correlation with serum T4S ($R=-0.53$, $P<0.001$) and T4S/T4 ratio ($R=-0.62$, $P<0.001$). Cause of death was significantly correlated with hepatic T2- and E2-SULT activities ($P<0.01$), with SULT activities being highest in the patients who died of severe brain damage and lowest in the patients who died of a cardiovascular collapse. A longer period of intensive care was associated with higher levels of T4S ($P=0.005$), and high levels of bilirubin were associated with low T2-SULT ($P=0.04$) activities and high levels of T4S ($P<0.001$).

**Conclusion:** Serum T4S levels were clearly elevated compared to healthy references, and the decreased deiodination by liver D1 during critical illness appears to play a role in this increase in serum T4S levels.

## Introduction

During critical illness, pronounced alterations in the hypothalamus-pituitary-thyroid axis occur without any evidence for thyroid disease (1-4). Decreased plasma $T_3$ and increased plasma $rT_3$ levels are the most common changes, and the magnitude of these changes is related to the severity of the disease (1, 2, 5). This combination of decreased serum $T_3$ and increased serum $rT_3$ levels indicates major changes in the peripheral metabolism of thyroid hormone, which is mediated importantly by the three iodothyronine deiodinases D1, D2 and D3 (see Refs (5, 6) for reviews). Reduced activity of D1 and increased activity of D3 have been shown in post-mortem liver and skeletal muscle tissues of non thyroidal illness (NTI) patients (1, 7).

The role of sulfation in thyroid hormone metabolism is fascinating. Sulfated iodothyronines do not bind to the thyroid hormone receptors, and sulfation medi-

ates the rapid and irreversible degradation of iodothyronines by D1 (8). Therefore, the concentrations of sulfated iodothyronines in serum are normally low (9-12). Inner ring deiodination (inactivation) of T4 and T3 by D1 is markedly facilitated after sulfation, whereas outer ring deiodination of T4 is blocked after sulfation (13-15). D2 and D3 are incapable of catalyzing the deiodination of sulfated iodothyronines (15).

Iodothyronine sulfation is catalyzed by cytosolic sulfotransferases (SULT) using 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as the sulfate donor (16-18). The sulfotransferases show overlapping substrate specificity. In humans, they can be subdivided into different families, SULT1, SULT2, SULT4, and SULT6(19) (20). It has been shown that all members of the human SULT1 family, i.e. hSULT1A1, 1A2, 1A3, 1B1, 1C2, 1C4, and 1E1, catalyze the sulfation of iodothyronines (21-24). hSULT1A1-3, 1B1, 1C4, and also native enzymes in liver have a substrate preference for 3,3'-diiodothyronine (T2), which is catalyzed much faster than T3 and rT3, whereas T4 sulfation is negligible (20, 23, 24). hSULT1E1 equally prefers T2 and rT3 over T3 and T4, but is the only known SULT so far having significant T4 sulfotransferase activity (23).

Elevated T4S levels and T3S/T3 ratios have been reported in NTI patients, and T4S levels are increased in preterm infants (11, 12, 25, 26). In this study we investigated if T4S levels are increased in patients who died in the intensive care unit (ICU), and if T4S levels are correlated with liver D1 or SULT activity. Furthermore, we investigated if T4S levels or SULT activities were regulated by different parameters of disease during critical illness. All patients in this study had been randomised for insulin treatment, as part of a large clinical trial that was published in detail elsewhere (27).

## Patients and Methods

### *Patients*

This study was part of a large randomized controlled study on intensive insulin treatment in ICU patients (N=1548), of which the major clinical outcomes have been published in detail elsewhere (27). All mechanically ventilated patients were eligible for inclusion in this trial after informed consent from the closest family member. On admission, patients were randomly assigned to either strict normalization of blood glucose (80-110 mg/dl) with intensive insulin therapy or the conventional approach, in which insulin infusion is initiated only when blood glucose exceeds 215 mg/dl, to maintain blood glucose levels between 180 and 200 mg/dl.

The study protocol has been approved by the Ethical Review Board of the University of Leuven School of Medicine.

A total of 79 patients were included in the current study. All patients included had died in the ICU, and the cause of death was determined both clinically by the attending ICU physician and by post-mortem examination. The pathologist was unaware of insulin treatment allocation. Relevant patients' characteristics are summarized in Table 1a.

**Table 1a.** Descriptive statistics of the population investigated in this study

| | |
|---|---|
| No. of patients | 79 |
| Age (years) | 68.6 ± 12.1 |
| No. of male patients (%) | 55 (69.6 %) |
| BMI (kg/m$^2$) | 25.4 ± 4.7[a] |
| ICU stay (days) | 13 (6-34) |
| No. of patients receiving TH treatment (%) | 31 (39.2%) |
| Serum T4 (nmol/L) | 40.6 (24.2-67.1)[b] |
| Serum T4S (pmol/L) | 138 (71-282)[b] |
| Serum T3 (nmol/L) | 0.98 (0.68-1.57)[b] |
| Serum rT3 (nmol/L) | 1.37 (0.67-2.31)[b] |
| Liver D1 (pmol/mg/min) | 3.25 (1.64-6.31)[c] |
| Liver T2 SULT (pmol/mg/min) | 14.72 (10.69-18.90)[c] |
| Liver E2 SULT (fmol/mg/min) | 30.60 (16.12-42.77)[d] |

[a] N= 78, [b] N= 64, [c] N= 65, [d] N= 57, TH, Thyroid Hormone; IQR, Interquartile range
Age and BMI are shown as mean ± SD, ICU stay, serum measurements, and D1 and SULT activities are shown as median (IQR).

Blood samples were obtained from 64 patients and liver biopsies from 65 patients within minutes after death. From 50 patients, both serum and liver samples were available. From an additional 14 patients, only a liver sample was available, and from 15 other patients, only serum was available. The total number of patients in this study was therefore 79. All patients had been randomized for conventional or intensive insulin treatment (27). 31 patients had been treated with thyroid hormone during the course of their critical illness. Treatment was initiated when they had a serum T4 concentration below 50 nmol/l in the face of a normal thyroxine-binding globulin and concomitantly clinical symptoms of hypothyroidism (7). In these

cases, thyroid hormone treatment consisted of an IV bolus of 150 µg T4 daily plus 0.6 µg T3 per kg body weight per 24 h as a continuous IV infusion.

### *Serum analyses*

Serum total T4, total T3, and TSH were measured by chemoluminescence assays (Vitros ECi Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK). rT3 was measured by radioimmunoassay as previously described (28). $T_4S$ was prepared by the method of Eelkman Rooda et al. (29). Serum $T_4S$ was measured using a specific antibody, as described previously (30). Within assay coefficients of variation amounted to 4% for TSH, 2% for T4, 2% for T3, 3-4% for rT3, and 6-17% for T4S. Normal values (mean ± 2xSD) for T4S were determined in 172 healthy individuals.

### *D1 and sulfotransferase activities*

Human liver and muscle samples were homogenized on ice in 10 volumes of PE buffer (0.1 M phosphate, 2 mM EDTA, pH 7.2) using a Polytron (Kinematica AG, Lucerne, Switzerland). Homogenates were snap-frozen in aliquots and stored at -80 C until further analysis. Protein concentration was measured with the Bio-Rad Protein Assay using BSA as the standard following the manufacturer's instructions.

Liver D1 activities were determined as described earlier (31) by duplicate incubations of homogenates (10 µg protein) for 30 min at 37 C with 0.1 µM [3',5'-$^{125}$I]rT3 (100,000 cpm) in a final volume of 0.1 ml PE buffer with 10 mM DTT). Reactions were stopped by addition of 0.1 ml 5% (wt/vol) BSA in water on ice. The protein-bound iodothyronines were precipitated by addition of 0.5 ml ice-cold 10% (wt/vol) trichloroacetic acid in water. Following centrifugation, $^{125}I^-$ was isolated from the supernatant by chromatography on Sephadex LH-20 minicolumns (32).

T2 sulfotransferase (T2-SULT) activity was analyzed by incubation of 0.1 µmol/L 3,3-$T_2$ including 100,000 cpm $^{125}$I-labeled T2 for 30 min at 37 C with liver or muscle homogenate in the presence or absence (blank) of 50 µM PAPS in 0.2 mL PE buffer (33). The reactions were stopped by the addition of 0.8 mL 0.1 M HCl, and the mixtures were analyzed for sulfate formation by chromatography on Sephadex LH-20 minicolumns as previously described (33). Sulfation in reaction mixtures with PAPS was corrected for background radioactivity detected in the corresponding Sephadex LH-20 fractions of the blanks. Incubations were carried out in triplicate.

Estrogen sulfotransferase (E2-SULT) activity was analyzed by incubation of 1 nM $^{3}$H-labeled E2 for 30 min at 37 C with liver homogenate in the absence (blank) or presence of 50 μM PAPS in 0.2 ml PE buffer with 1 mM DTT (23). The reactions were stopped by addition of 0.8 ml ice-cold water, and the mixtures were extracted with 2.5 ml dichloromethane. Sulfate formation was quantified by counting 0.5 ml of the aequous phase. Enzymatic sulfation was adjusted for background radioactivity estimated in the blanks.

### *Statistical analysis*

Data were analyzed using the statistical program SPSS 10.0.7 for Windows (SPSS, Chicago, IL). Logarithmic transformations were applied in order to normalize variables and to minimize the influence of outliers, when appropriate. All analyses were done on the whole group, as well as on subgroups treated or not treated with thyroid hormone. Data were analyzed using one-way ANOVA tests, with a post-hoc Fisher's least significant difference (LSD) test for multiple comparisons, Mann-Whitney U tests, and linear regression analyses, when appropriate. Correlation coefficients represent Spearman's correlation coefficients. Statistical significance was assumed for $P < 0.05$.

## Results

### *Insulin therapy did not affect serum T4S levels and hepatic SULT activities*

The baseline characteristics of the patients who were treated with conventional or intensive insulin therapy are shown in Table 1a. There was no difference in age, gender, BMI, stay on the ICU, need for thyroid hormone treatment, or iodothyronine levels between the two treatment groups. Neither T4S, nor hepatic T2- and E2-SULT activities were different between the conventional and intensive insulin treatment groups.

Characteristics of the patients who were treated or not treated with thyroid hormone are shown in Table 1b. Serum T3 and T4S, but not serum T4 levels, were higher in patients who were treated with thyroid hormone (7) (Table 1b). T2- and E2-SULT activities were not different between the two treatment groups.

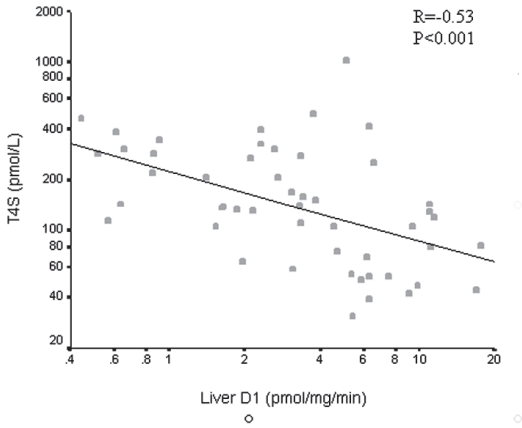
**Table 1b.** Descriptive statistics of the patients who were treated and who were not treated with thyroid hormone

| | TH treatment | | |
|---|---|---|---|
| | No | Yes | *P* |
| No. of patients | 48 | 31 | |
| Age (years) | 68.5 ± 13.6 | 68.8 ± 9.7 | 0.91 |
| No. of male patients (%) | 34 (70.8 %) | 21 (67.7 %) | 0.77 |
| Serum T4 (nmol/L)[a] | 38.8 (21.7-66.6) | 44.7 (26.1-71.4) | 0.50 |
| Serum T4S (pmol/L) [a] | 106 (55-169) | 223 (130-335) | **0.003** |
| Serum T3 (nmol/L) [a] | 0.79 (0.61-1.11) | 1.41 (0.98-2.03) | **<0.001** |
| Serum rT3 (nmol/L) [a] | 1.13 (0.43-2.02) | 1.85 (0.99-2.43) | 0.06 |
| Liver D1 (pmol/mg/min) [b] | 3.33 (1.70-6.29) | 2.55 (1.56-6.42) | 0.62 |
| Liver T2 SULT (pmol/mg/min) [b] | 14.75 (11.17-18.98) | 14.00 (9.52-18.73) | 0.35 |
| Liver E2 SULT (fmol/mg/min) [c] | 30.60 (16.12-46.87) | 29.76 (16.26-40.77) | 0.91 |

[a] N= 39 vs. 25, [b] N= 44 vs. 21, [c] N= 37 vs. 20
Age is shown as mean ± SD, serum measurements, and D1 and SULT activities are shown as median (IQR). P values represent Mann-Whitney U tests.
TH, Thyroid Hormone; IQR, Interquartile range

### *Correlation analysis of T4S with D1 and SULT activities*

Compared to the reference values used in our lab (T4S < 90 pmol/L) ([5.3 – 29.3 pmol/L], mean 17.3 pmol/L, SD 6.0 pmol/L, determined in 172 healthy references) and compared to values that have been described by others (19 ± 1.2 pmol/L, mean ± SE) (12), T4S levels were significantly elevated in the patients in this study. As described earlier, serum T4 and liver D1 activity were significantly lower than in healthy controls (7). Liver D1 activity showed a strong, negative correlation with serum T4S (R=-0.53, P<0.001) and with the T4S/T4 ratio (R=-0.62, P<0.001) (Fig. 1). T2-SULT activity was not correlated with serum T4S or the T4S/T4 ratio, nor was E2-SULT activity (Table 2).



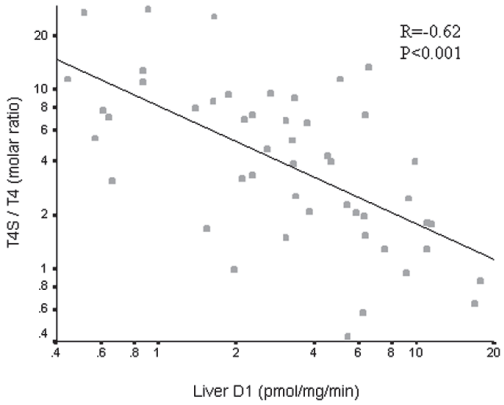




**Figure 1.** Correlation analysis of liver D1 activity and serum T4S (A) and T4S/T4 ratio (B). Liver D1 showed a significant negative correlation with T4S (P<0.001) and with T4S/T4 ratio (P<0.001). No distinction was made in this figure between patients who received thyroid hormone treatment and patients who did not. R represents Spearman's correlation coefficient.

**Table 2.** Correlation analysis of liver D1 and T2-SULT activity and serum thyroid hormone levels.

| | | D1 | T2-SULT | E2-SULT |
|---|---|---|---|---|
| T4 (nmol/L) | R | 0.34 | 0.13 | 0.20 |
| | P | **0.02** | 0.36 | 0.20 |
| T4S (pmol/L) | R | -0.53 | -0.06 | -0.20 |
| | P | **<0.001** | 0.70 | 0.21 |
| T3 (nmol/L) | R | 0.05 | -0.10 | -0.14 |
| | P | 0.71 | 0.47 | 0.37 |
| rT3 (nmol/L) | R | -0.65 | -0.26 | -0.53 |
| | P | **<0.001** | 0.07 | **<0.001** |
| T4S / T4 (molar ratio) | R | -0.62 | -0.08 | -0.27 |
| | P | **<0.001** | 0.57 | 0.09 |

R represents Spearman's Correlation coefficient,
N=50 for D1 and T2-SULT, and N=43 for E2-SULT

Similar to hepatic D1 activity (7), high E2-SULT activity was associated with low serum rT3 levels, and T2-SULT activities showed a negative trend with serum rT3 levels (Table 2). Hepatic D1 was positively correlated with T2-SULT activity (R=0.29, P=0.02) and with E2-SULT activity (R=0.39, P=0.003). The association of E2-SULT activity with serum rT3 was not entirely caused by the positive relation of E2-SULT and D1 activity, since a trend towards lower rT3 levels remained in a multivariate analysis (P=0.07 for D1 and P=0.08 for E2-SULT activity). Furthermore, T2- and E2-SULT activities were positively correlated (R=0.65, P<0.001).

### *Relation of T4S and SULT activities and cause of death*

Similar to hepatic D1 activity (7), hepatic T2- and E2-SULT activities showed a significant correlation with cause of death, being lowest in the patients who had died of a cardiovascular collapse, with successive increases in the patients who had died of multiple organ failure (MOF) with sepsis or MOF with systemic inflammatory response syndrome (SIRS), and being highest in the patients who had died of severe brain damage (ANOVA P=0.001 for T2-SULT and P=0.002 for E2-SULT activity) (Fig. 2A and B). Serum T4S levels did not correlate with cause of death (Fig. 2C).

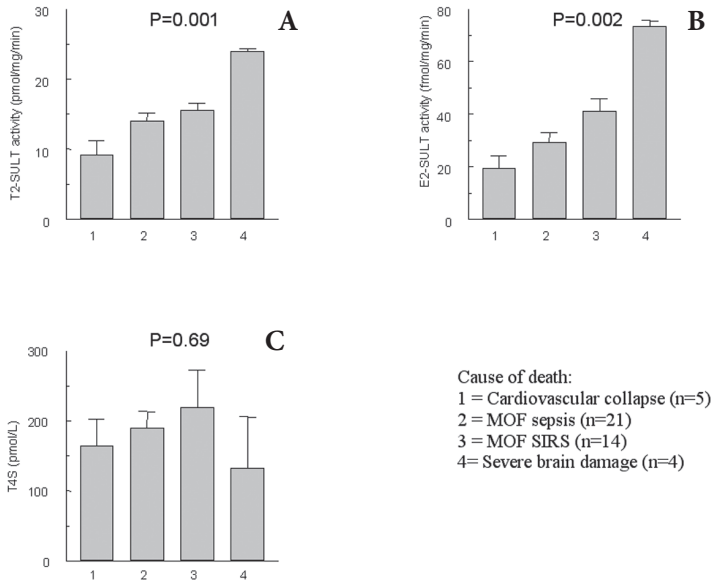



**Figure 2.** Correlation of hepatic T2- and E2-SULT activities and of serum T4S levels with cause of death. Patients were divided in four different groups based on cause of death. I = Cardiovascular collapse (n=5), II = MOF sepsis (n=21), III = MOF SIRS (n=14), IV = Severe brain damage (n=4). Hepatic T2- and E2-SULT activities, but not serum T4S levels showed a significant relation with cause of death (P<0.01). P-values are for trends. Data represent means ± SEM.

*Relation of T4S and SULT activities with ICU stay, liver and kidney function*

Although the number of days that a patient received intensive care before he/she died was not correlated with hepatic D1 (P=0.45), T2-SULT (P=0.21), and E2-SULT activities (P=0.70), length of ICU stay was correlated with higher levels of T4S (Fig. 3). High plasma levels of total bilirubin on the last day were associated with low hepatic T2-SULT activity (Fig. 4A) and D1 activity (7), and with high levels of serum T4S (Fig. 4B). E2-SULT activity was not correlated with plasma bilirubin levels (P=0.34). Plasma urea and plasma creatinin on the last day were not associated with serum T4S or hepatic SULT activities (data not shown).

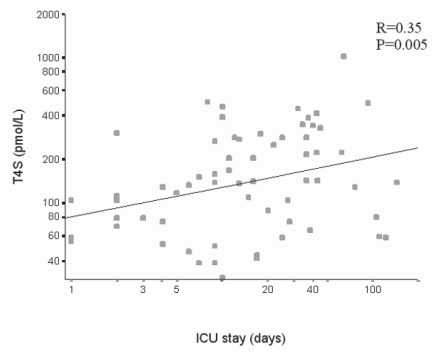



**Figure 3.** Correlation analysis of serum T4S levels and the number of days that a patient received intensive care. The number of days were positively correlated with T4S levels (P<0.001). No distinction was made in this figure between patients who received thyroid hormone treatment and patients who did not. R represents Spearman's correlation coefficient.

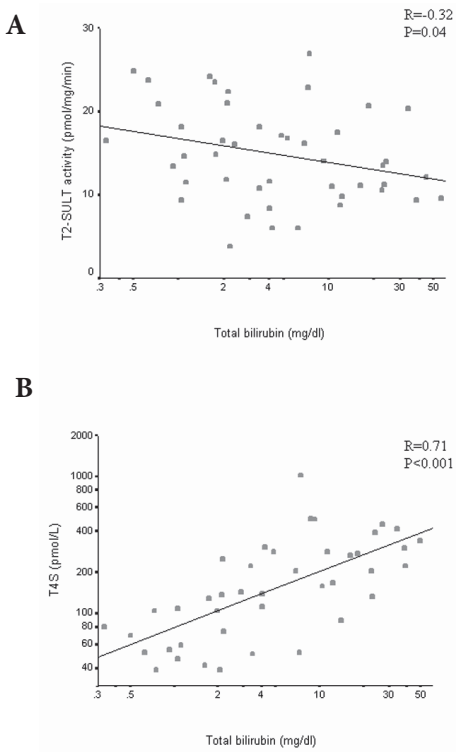



**Figure 4.** Correlation analysis of hepatic T2-SULT activity (A) and serum T4S levels with plasma total bilirubin levels on the last day. T2-SULT activity showed a significant negative correlation with bilirubin levels (P=0.04), whereas T4S levels were positively correlated (P<0.001). No distinction was made in this figure between patients who received thyroid hormone treatment and patients who did not. R represents Spearman's correlation coefficient.

## Discussion

No differences in serum T4S or hepatic SULT activities were found between critically ill patients who were treated with intensive or with conventional insulin therapy. In these critically ill patients, T4S levels were significantly elevated compared to healthy references. Furthermore, hepatic D1 showed a strong negative correlation with serum T4S and T4S/T4 ratio, whereas SULT activities were not correlated with these parameters. Cause of death was significantly correlated with hepatic T2- and E2-SULT activities, the latter being highest in the patients who died of severe brain damage and lowest in the patients who died of a cardiovascular collapse. A longer period of intensive care was associated with higher levels of T4S, and high levels of bilirubin were associated with low T2-SULT activities and high levels of T4S.

Insulin therapy did not affect serum T4S or hepatic SULT activities, which is in agreement with previous studies in which we found no effect of insulin therapy on serum thyroid hormone levels and tissue deiodinase activities (7, 34). It should be noted, however, that the current study involved post-mortem samples. Since insulin therapy has a beneficial effect on both morbidity and mortality (27), the possibility of a selection bias (sicker patients in the studied group receiving intensive insulin therapy), cannot be excluded.

Despite low levels of serum T4, T4S levels were increased in these patients, which can be due to an increased production and/or a decreased clearance. D1 is the enzyme responsible for the breakdown of T4S, and in previous studies it has been shown that critical illness is associated with a decreased D1 activity (1, 5, 7, 20, 34). The strong negative correlation of hepatic D1 activity with serum T4S and with the T4S/T4 ratio suggests that a decreased liver D1 activity plays an important role in the increase of T4S levels during critical illness (12). Interestingly, T4S levels were highest in patients who were treated with thyroid hormone. Treatment consisted of a combination of T4 and T3, and patients who were treated had higher levels of T3 and similar levels of T4 compared to patients who were not treated. Although T3 is known to stimulate D1 expression, D1 activities were similar between the two treatment groups, suggesting that other factors than D1 contributed to the increased T4S levels in patients treated with thyroid hormone. There might have been a greater availability of T4 in these patients, although serum T4 levels were not significantly different from patients who were not treated with thyroid hormone. Since there was also no difference in SULT activities between the two groups, a low expression of transporters such as Na-taurocholate cotransporting polypeptide and different organic anion transporter polypeptides, which mediate the hepatic uptake of iodothyronine sulfates, may be an explanation (35-37).

T4-SULT activity in liver is low, and it is therefore not possible to measure T4-SULT activity directly in liver homogenates (24). Since the sulfotransferases show overlapping substrate specificity, and T2 is the preferred substrate for native liver sulfotransferases (20, 23, 24), we used T2, and not T4, as a substrate in our experiments. hSULT1E1 is the only known SULT so far catalyzing T4 sulfation, although it equally prefers T2 and rT3 over T3 and T4 (23). Because this enzyme has a much higher affinity for estrogens than for iodothyronines, we also measured hSULT1E1 using E2 as a substrate. No relation of hepatic T2- and E2-SULT activity was observed with serum T4S or with the T4S/T4 ratio. Obviously, this does not exclude a relation of in vivo T4-SULT activity and serum T4S levels.

All SULTs use PAPS as the sulfate donor (16-18). This makes sulfation expensive in terms of cellular energy, since two molecules of ATP are required to synthesize one molecule of PAPS (38, 39). In this study, SULT activities were determined in the presence of excess of exogenous cofactor. This study therefore does not provide any information about changes in the natural availability of PAPS in critical illness, when most patients have a negative energy balance, as a possible mechanism for regulation of SULT activity. Similarly, changes in the availability of $SO_4^{2-}$ during critical illness may also regulate SULT activities in these patients.

T2- and E2-SULT activities were significantly correlated with cause of death, in a pattern similar to hepatic D1 activity (7), and were not correlated with length of ICU stay. In a previous study we assumed that liver D1 expression in patients who died acutely from severe brain damage approximates that in healthy subjects (7). This was supported by the findings that liver D1 expression of these patients was similar to D1 activity observed in normal liver obtained from patients undergoing surgical removal of hepatic tumors. In this study, T2- and E2-SULT activities were also highest in patients who died acutely of severe brain damage. T2-SULT activities were in the same order of magnitude as we observed previously in cytosols of normal liver (24), suggesting a down-regulation of T2- and E2-SULT activities during critical illness. This low expression of hepatic SULTs during critical illness may have contributed to the increased rT3 levels in these critically ill patients, since hSULT1E1 is by far the best SULT for rT3 sulfation, and sulfation of E2 is highly correlated to sulfation of rT3 in human liver (23) (unpublished observations). In this study, low hepatic SULT activities were associated with high serum rT3 levels, independent of liver D1 activity. Nevertheless, it might be that other factors such as severity of disease, which may affect both E2-SULT activity and serum rT3 levels in parallel, cause this effect.

A longer period of intensive care was associated with higher levels of T4S, suggesting that also the duration of disease may be important in the increase in T4S during critical illness. High levels of bilirubin were associated with a low T2-SULT and D1 activity (7), and with high T4S levels. An impaired liver function may not only result in a lower sulfation of iodothyronines, but also in a decreased degradation of the sulfated iodothyronines, with the net effect being an increase in T4S levels. There was no such a relation for high levels of urea and creatinine, suggesting that the relation was specific for liver and not caused by a more general organ failure.

In conclusion, serum T4S levels in critically ill patients were clearly elevated compared to normal values. This study demonstrates that the decreased activity of liver D1 during critical illness appears to play an important role in the increase in serum T4S levels, since there was a strong negative correlation of hepatic D1 activity with serum T4S and with the T4S/T4 ratio, whereas hepatic SULT activities were not correlated with these parameters.

## Acknowledgements

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# *Chapter B4*

## Tissue thyroid hormone levels in critical illness

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## Abstract

Pronounced alterations in serum thyroid hormone levels occur during critical illness. Triiodothyronine (T3) decreases and reverse T3 (rT3) increases, the magnitudes of which are related to the severity of disease. It is unclear whether these changes are associated with decreased tissue T3 concentrations and, thus, reduced thyroid hormone bioactivity. We therefore investigated in 79 patients who died after intensive care and who did or did not receive thyroid hormone treatment, if total serum thyroid hormone levels correspond to tissue levels in liver and muscle. Furthermore, we investigated the relation between tissue thyroid hormone levels, deiodinase activities, and monocarboxylate transporter 8 (MCT8) expression. Tissue iodothyronine levels were positively correlated with serum levels, indicating that the decrease in serum T3 during illness is associated with decreased levels of tissue T3. Higher serum T3 levels in patients who received thyroid hormone treatment were accompanied by higher levels of liver and muscle T3, with evidence for tissue specific regulation. Tissue rT3 and the T3/rT3 ratio were correlated with tissue deiodinase activities. MCT8 expression was not related to the ratio of the serum over tissue concentration of the different iodothyronines. Our results suggest that, in addition to changes in the hypothalamus-pituitary-thyroid axis, tissue-specific mechanisms are involved in the reduced supply of bioactive thyroid hormone in critical illness.

## Introduction

Pronounced alterations in the hypothalamus-pituitary-thyroid axis occur during critical illness without any evidence for thyroid disease (1, 2). Total plasma triiodothyronine (T3) decreases and plasma reverse T3 (rT3) increases within a few hours after the onset of disease, and the magnitude of these changes is related to the severity of the disease (2-5). In severely ill patients, thyroxine (T4) decreases as well and both T4 and T3 are inversely correlated with mortality rate (2, 6). Hardly any data are available in humans whether these changes in serum thyroid hormone levels also result in changes in (free) tissue concentrations (7), and thus in a decreased bio-activity of thyroid hormone.

It is still controversial whether the reduction in serum T3 is a beneficial adaptation resulting in a protection against catabolism, or that it is a mal-adaptation contributing to a worsening of the disease (2, 8, 9). It has not been clearly demonstrated that substitution of critically ill patients with thyroid hormone has a positive

effect on clinical outcome (10-12), and it is unclear if thyroid hormone is taken up and metabolized in tissues when patients are treated with T4 and/or T3.

The decrease in serum T3 and the increase in rT3 levels can (partially) be explained by a reduced activation of T4 by type I deiodinase (D1) or D2, but also by an increased inactivation by D3 (1, 13). There is also evidence that next to the regulation of D1, D2, and D3, a diminished transport of thyroid hormone into D1 expressing tissues plays a role in the changes in serum iodothyronines levels during critical illness (5, 14).

In this study, we investigated in a group of patients who died after intensive care if local thyroid hormone levels in liver and skeletal muscle correspond to serum thyroid hormone levels. Furthermore, we investigated the relation between tissue thyroid hormone levels, deiodinase activities, and monocarboxylate transporter 8 (MCT8), a recently identified thyroid hormone specific transporter (15), expression in liver and skeletal muscle. All patients in this study had been randomized for insulin treatment, as part of a large clinical trial that was recently described elsewhere (16).

## Patients and Methods

### *Patients*

This study was part of a large randomized controlled study on intensive insulin treatment in intensive care unit (ICU) patients (N=1548), of which the major clinical outcomes have been published in detail elsewhere (16). All mechanically ventilated adult patients were eligible for inclusion in this trial after informed consent from the closest family member. The study protocol has been approved by the Ethical Review Board of the Catholic University of Leuven School of Medicine.

A total of 79 patients were included in the current study. All these patients had died on the ICU, and the cause of death was determined both clinically by the attending ICU physician and by post-mortem examination. 11 patients died of a cardiovascular collapse, 36 died of multi-organ failure (MOF) with sepsis, 26 died of MOF with systemic inflammatory response syndrome, and 6 died of severe brain damage. Relevant patients' characteristics are summarized in Table 1.

Blood samples were obtained from 64 patients, open liver biopsies from 65 patients, and open skeletal muscle (rectus abdominis) biopsies from 65 patients within minutes after death. The time between death and obtaining the tissue was 20 [12-31] min (median [interquartile range]) for liver and 17 [8-25] min for skeletal muscle. From 49 patients blood, liver as well as skeletal muscle samples were available. 31 patients had been treated with thyroid hormone during the course of their

critical illness. Treatment was initiated when they had a serum T4 concentration below 50 nmol/l in the face of a normal thyroxine-binding globulin and concomitantly clinical signs of hypothyroidism. The latter was defined as coma or central nervous system suppression, failure to wean from the ventilator, or hemodynamic instability, which were unexplained and resistant to conventional supportive therapy. In these cases, thyroid hormone treatment consisted of an IV bolus of 150 μg T4 daily plus 0.6 μg T3 per kg body weight per 24 h as a continuous IV infusion. The median treatment was 11 days (6-23 IQR).

**Table 1.** Descriptive statistics of the population used in this study

| | Conventional Insulin treatment | Intensive Insulin treatment | *P* |
|---|---|---|---|
| No. of patients | 54 | 25 | |
| Age (years) | 68.6 ± 12.1 | 68.5 ± 12.4 | 0.68 |
| No. of male patients (%) | 37 (68.5 %) | 18 (72.0 %) | 0.75 |
| ICU stay (days) | 16 (7-36) | 11 (4-25) | 0.25 |
| No. of patients receiving TH (%) | 19 (35.2%) | 12 (48.0%) | 0.28 |
| No. of patients receiving glucocorticoids (%) | 28 (51.9%) | 14 (56%) | 0.73 |
| No. of patients receiving dopamine (%) | 20 (37.0%) | 9 (36.0%) | 0.93 |
| Serum TSH (μU/mL) | 0.21 (0.04-0.96) [b] | 0.05 (0.003-0.57) [b] | 0.08 |
| Serum T4 (μg/dL) | 3.35 (1.73-5.10)[b] | 2.91 (1.93-5.98)[b] | 0.66 |
| Serum T3 (ng/dL) | 62.30 (44.13-99.95)[b] | 73.99 (43.48-114.22)[b] | 0.64 |
| Serum rT3 (ng/dL) | 98.65 (46.08-151.87)[b] | 88.91 (27.91-133.05)[b] | 0.48 |
| Liver D1 (pmol/mg/min) | 3.25 (1.63-6.19)[c] | 3.21 (1.64-7.63)[c] | 0.73 |
| Liver D3 (fmol/mg/min) | 0.60 (0.41-1.09)[c] | 0.62 (0.42-1.06)[c] | 0.91 |
| Muscle D3 (fmol/mg/min) | 0.14 (0.11-0.27)[d] | 0.13 (0.09-0.19)[d] | 0.24 |
| Liver T4 (ng/g) | 36.05 (17.33-71.95)[c] | 23.47 (9.32-36.29)[c] | 0.13 |
| Liver T3 (ng/g) | 0.96 (0.64-2.75)[c] | 1.17 (0.62-3.03)[c] | 0.57 |
| Liver rT3 (ng/g) | 1.77 (1.05-5.14)[c] | 1.32 (0.84-2.88)[c] | 0.10 |
| Muscle T4 (ng/g) | 1.90 (1.23-2.96)[e] | 2.18 (1.34-2.87)[d] | 0.64 |
| Muscle T3 (ng/g) | 0.23 (0.18-0.36)[d] | 0.28 (0.21-0.42)[d] | 0.33 |
| Muscle rT3 (ng/g) | 0.64 (0.42-1.15)[d] | 0.57 (0.34-0.79)[d] | 0.21 |

[b] N= 48 vs. 16, [c] N= 43 vs. 22, [d] N= 42 vs. 23, [e]N= 41, [f] N= 37 vs. 20, [g] N= 42 vs. 23
Age and BMI are shown as mean ± SD, ICU stay, other data are shown as median (IQR).
***P*** values represent Mann-Whitney U tests.
TH, Thyroid Hormone; IQR, Interquartile range

### *Serum analyses*

The care of patients in the ICU often comprises infusion of heparin, either systemically or locally to prevent clotting of vascular access, which substantially interferes with the assay used to quantify free concentrations of thyroid hormone (17). Therefore, we refrained from measuring serum FT4 and FT3 in this study. Serum total T4, T3, rT3 and TSH were measured as previously described (13, 18). Normal values for TSH (0.4 – 4.3 μU/dL), T4 (4.51-9.95 μg/dL), T3 (92.8-162.9 ng/dL) and rT3 (9.1-22.1 ng/dL) were determined in 270 healthy individuals. TBG levels were measured using a commercially available radioimmunoassay (Schering-CIS Biointernational, Gif-sur-Yvette, France).

### *Determination of T4, T3, and rT3 concentrations in human liver and skeletal muscle samples*

T4, T3, and rT3 were determined by highly sensitive and specific RIAs after extraction and purification of the iodothyronines from tissues, as described elsewhere (19, 20). 2000 cpm [$^{131}$I]T4 and [$^{125}$I]T3 were added to each sample as internal tracers for recovery calculations. Average recovery was 50.6 % and 55.4 % % for [$^{131}$I]T4 in liver and skeletal muscle respectively, and 69.0 % and 72.3 % for [$^{125}$I]T3. Due to the limited amount of available tissue, [$^{125}$I]T4 was also used as a recovery tracer for the determination of rT3, since previous experiments in our lab have shown that recovery of T4 and rT3 is similar. No corrections for the amounts of iodothyronines contributed by the blood trapped in the tissue aliquot were carried out.

### *Tissue deiodinase activities*

Human liver and skeletal muscle samples were homogenized on ice in 10 volumes of PE buffer (0.1 M phosphate, 2 mM EDTA, pH 7.2) using a Polytron (Kinematica AG, Lucerne, Switzerland). Homogenates were snap-frozen in aliquots and stored at -80 C until further analysis. Liver and skeletal muscle deiodinase activities were determined as described earlier (13).

### *RNA isolation and reverse transcription*

RNA was isolated from liver samples using the High Pure RNA Tissue Kit (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's protocol. With this method, RNA is bound to a glass fiber fleece, and eluted with distilled water. RNA isolation from skeletal muscle failed, because skeletal muscle tissue obstructed the fleece. Therefore, skeletal muscle RNA was isolated using Trizol reagent

(GIBCO BRL, Breda, The Netherlands) as an initial isolation step, with a further purification of the RNA using the High Pure RNA Tissue Kit. RNA concentrations were determined using the RiboGreen® RNA Quantitation Kit (Molecular Probes, Leiden, The Netherlands). All samples were diluted to 0.1 µg/µl, and 1 µg was used for cDNA synthesis using the TaqMan™ Reverse Transcription Kit (Roche Diagnostics).

### *Real-time RT- PCR*

MCT8 mRNA levels were determined in 57 liver samples and 65 skeletal muscle samples using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands), which employs TaqMan™ chemistry for highly accurate quantitation of mRNA levels, as previously described (13). Sequences and concentrations of the primers and probes are given in Table 2. mRNA levels are expressed relative to those of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene. The GAPDH probe and primers were provided as pre-optimized control system (Applied Biosystems).

**Table 2.** Sequences and concentrations of the primers and probe that were used for determination of MCT8 mRNA levels by quantitative real-time PCR

| | Sequence | Concentration |
|---|---|---|
| MCT8-fwd | 5'-CCATAACTCTGTCGGGATCCTC-3' | 300 nmol/L |
| MCT8-probe | 5'-FAM-ATACCCATCGCGAGGGCTCCGA-TAMRA-3' | 200 nmol/L |
| MCT8-rev | 5'-ACTCACAATGGGAGAACAGAAGAAG-3' | 300 nmol/L |

Data were expressed as Ct values, which represent the cycle number at which probe-derived dye absorbance reaches the calculated treshold value. Data are expressed relative to the housekeeping gene as ($2^{\Delta Ct}$) x 1000. ΔCt is the Ct value of the housekeeping gene minus the Ct value of the target gene.

### *Statistical analysis*

Data were analyzed using the statistical program SPSS 10.0.7 for Windows (SPSS, Chicago, IL). Logarithmic transformations were applied in order to normalize variables and to minimize the influence of outliers, when appropriate. All analyses were done on the whole group, as well as on subgroups treated or not treated with thyroid

hormone. Data were analyzed using one-way ANOVA tests, with a post-hoc Fisher's least significant difference (LSD) test for multiple comparisons, Mann-Whitney U tests, and linear regression analyses, when appropriate. Correlation coefficients represent Spearman's correlation coefficients. Statistical significance was assumed for $P < 0.05$.

## Results

*The effect of insulin and thyroid hormone therapy on serum and/or tissue thyroid parameters*

The baseline characteristics of the two insulin treatment groups are shown in Table 1. There was no difference in age, gender, length of intensive care, need for thyroid hormone treatment, or any of the serum or tissue thyroid parameters between the two treatment groups. In both groups, iodothyronine concentrations in liver were substantially higher than in skeletal muscle. Table 3 shows the same characteristics for the patients who did and those who did not receive thyroid hormone treatment. No distinction can be made in this study between primary and illness-induced (central) hypothyroidism, but presumably most thyroid hormone-treated patients belonged to the latter category (1, 2, 9). Patients who were treated with thyroid hormone stayed longer on the ICU ( 25 [18-42] (median [IQR]) vs 9 [3-16]), received glucocorticoid treatment more often, and had a significantly lower TSH (0.01 [0.002-0.08] vs 0.61 [0.13-0.96]) and higher T3 (1.41 [0.98-2.03] vs 0.79 [0.61-1.11]) than patients who did not receive thyroid hormone treatment. T3 levels in patients who were treated with thyroid hormone were still in the low or low-normal range (Table 3). Higher serum T3 levels in these patients were accompanied by higher levels of liver T3 and rT3, higher levels of muscle T4, T3, and rT3, and by a higher muscle D3 activity. The increased liver T3 concentration in patients who received thyroid hormone treatment was remarkable, since it was disproportional compared to the increased serum and muscle T3 concentrations (~ 4 times higher in liver compared to ~2 times higher in serum and skeletal muscle).

*Correlation analysis of serum and tissue iodothyronine levels*

Serum T4, T3, and rT3 levels in serum showed a strong positive correlation with the respective hormone concentrations in liver ($R=0.31$, $P=0.03$; $R=0.81$, $P<0.001$; $R=0.58$, $P<0.001$ for T4, T3, and rT3, respectively), independent of thyroid hormone treatment (Table 4 and Figure 1). Similarly, skeletal muscle iodothyronine concentrations were positively correlated with serum iodothyronine concentrations

(R=0.67, P<0.001; R=0.72, P<0.001; R=0.71, P<0.001) (Table 4 and Figure 2). The relation between serum and tissue iodothyronine concentrations was weakest for liver T4, compared to the other iodothyronines in liver and skeletal muscle.

**Table 3.** Differences between the patients who were treated with thyroid hormone and the patients who were not

| | TH treatment = no | TH treatment = yes | ***P*** |
|---|---|---|---|
| No. of patients | 48 | 31 | |
| Age (years) | 68.5 ± 13.6 | 68.8 ± 9.7 | 0.58 |
| No. of male patients (%) | 34 (70.8 %) | 21 (67.7 %) | 0.77 |
| No. of patients receiving glucocorticoids (%) | 17 (35.4 %) | 25 (80.6%) | **<0.001** |
| No. of patients receiving dopamine (%) | 17 (35.4 %) | 12 (38.7 %) | 0.77 |
| ICU stay (days) | 9 (3-16) | 28 (18-43) | <0.001 |
| Serum TSH (μU/mL) | 0.61 (0.13-0.96) [b] | 0.01 (0.002-0.08) [b] | <0.001 |
| Serum T4 (μg/dL) | 3.01 (1.69-5.17)[b] | 3.47 (2.03-5.55)[b] | 0.50 |
| Serum T3 (ng/dL) | 51.27 (39.59-72.04)[b] | 91.51 (63.60-131.75)[b] | <0.001 |
| Serum rT3 (ng/dL) | 73.34 (27.91-131.10)[b] | 120.07 (64.25-157.71)[b] | 0.06 |
| Liver D1 (pmol/mg/min) | 3.33 (1.70-6.29)[c] | 2.55 (1.56-6.42)[c] | 0.62 |
| Liver D3 (fmol/mg/min) | 0.51 (0.36-1.04)[c] | 0.71 (0.47-1.13)[c] | 0.16 |
| Muscle D3 (fmol/mg/min) | 0.13 (0.09-0.23)[d] | 0.16 (0.13-0.26)[d] | 0.04 |
| Liver T4 (ng/g) | 23.47 (10.10-61.77)[d] | 29.99 (21.83-71.64)[d] | 0.14 |
| Liver T3 (ng/g) | 0.72 (0.54-1.26)[d] | 2.92 (0.99-3.71)[d] | **<0.001** |
| Liver rT3 (ng/g) | 1.38 (0.75-3.23)[d] | 2.76 (1.60-4.57)[d] | **0.01** |
| Muscle T4 (ng/g) | 1.63 (0.99-2.68)[e] | 2.29 (1.78-3.37)[f] | **0.01** |
| Muscle T3 (ng/g) | 0.22 (0.16-0.29)[f] | 0.36 (0.26-0.73)[f] | **<0.001** |
| Muscle rT3 (ng/g) | 0.48 (0.35-0.92)[f] | 0.75 (0.55-1.10)[f] | **0.02** |

[b] N= 39 vs. 25, [c] N= 44 vs. 21, [d] N= 43 vs. 22, [e]N= 41, [f] N= 42 vs. 21, [g] N= 36 vs. 21, [h] N= 42 vs. 22
Age and BMI are shown as mean ± SD, ICU stay, other data are shown as median (IQR).
P values represent Mann-Whitney U tests.
TH, Thyroid Hormone; IQR, Interquartile range

## *Correlation of tissue deiodinase activities and tissue iodothyronine levels*

As described previously, we were not able to measure any D2 activity in the liver and skeletal muscle samples of these patients, whereas D3 activity was induced in both liver and skeletal muscle (13). Liver D1 was negatively correlated with serum rT3 levels and positively with the serum T3/rT3 ratio. Liver D3 was positively corre-

lated with serum rT3 levels, whereas muscle D3 activity was not correlated to serum rT3 levels in these patients (13). No significant correlation was observed between deiodinase activities and post-mortem time.

**Table 4.** Correlation analysis of serum and tissue (liver and skeletal muscle) iodothyronine levels

| | TH treatment | | Liver | | | Muscle | | |
|---|---|---|---|---|---|---|---|---|
| | | | T4 | T3 | rT3 | T4 | T3 | rT3 |
| | All (N=51) | R | 0.31 | 0.38 | -0.09 | 0.67[a] | 0.38 | 0.08 |
| | | P | 0.03 | 0.006 | 0.53 | <0.001 | 0.006 | 0.60 |
| Serum T4 (μg/dL) | No (N=35) | R | 0.35 | 0.53 | -0.04 | 0.75[a] | 0.44 | 0.19 |
| | | P | 0.04 | 0.001 | 0.82 | <0.001 | 0.008 | 0.28 |
| | Yes (N=16) | R | 0.29 | 0.10 | -0.45 | 0.52[a] | 0.17 | -0.35 |
| | | P | 0.27 | 0.72 | 0.08 | 0.04 | 0.54 | 0.19 |
| | All (N=51) | R | 0.46 | 0.81 | 0.19 | 0.65[a] | 0.72 | 0.34 |
| | | P | 0.001 | <0.001 | 0.17 | <0.001 | <0.001 | 0.02 |
| Serum T3 (ng/dL) | No (N=35) | R | 0.45 | 0.72 | 0.06 | 0.65[a] | 0.50 | 0.28 |
| | | P | 0.006 | <0.001 | 0.74 | <0.001 | 0.002 | 0.11 |
| | Yes (N=16) | R | 0.24 | 0.73 | -0.11 | 0.41[a] | 0.85 | -0.13 |
| | | P | 0.37 | 0.001 | 0.70 | 0.11 | <0.001 | 0.64 |
| | All (N=51) | R | 0.22 | 0.25 | 0.58 | 0.23[a] | 0.32 | 0.71 |
| | | P | 0.12 | 0.08 | <0.001 | 0.11 | 0.02 | <0.001 |
| Serum rT3 (ng/dL) | No (N=35) | R | 0.33 | 0.11 | 0.64 | 0.16[a] | 0.14 | 0.65 |
| | | P | 0.05 | 0.54 | <0.001 | 0.36 | 0.43 | <0.001 |
| | Yes (N=16) | R | -0.35 | 0.24 | 0.24 | 0.20[a] | 0.26 | 0.70 |
| | | P | 0.18 | 0.37 | 0.36 | 0.46 | 0.34 | 0.003 |

R represents Spearman's Correlation coefficient, [a] N = 50, 34, 15, respectively

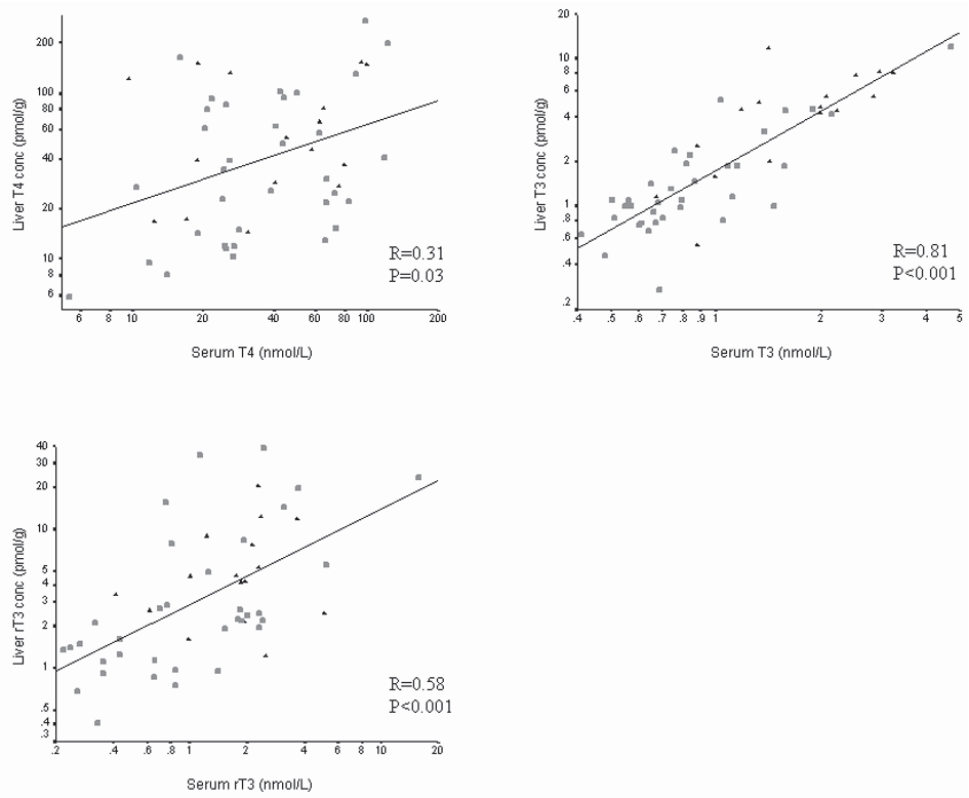


**Figure 1.** Correlation analysis of serum T4, T3, and rT3 levels with local T4, T3, and rT3 concentrations in liver. Patients who did not receive thyroid hormone treatment are represented by ●, whereas patients who received thyroid hormone therapy are represented by ▲. R represents Spearman's correlation coefficient. To convert values for serum T4 to μg/dL, multiply by 0.0777, and to convert values for serum T3 and rT3 to ng/dL, multiply by 64.9. Liver concentrations of T4, T3, and rT3 are shown in picomoles per gram wet weight. To convert values for liver T4 to nanograms per gram, divide by 1.287; to convert values for liver T3 and rT3 to nanograms per gram, divide by 1.54.

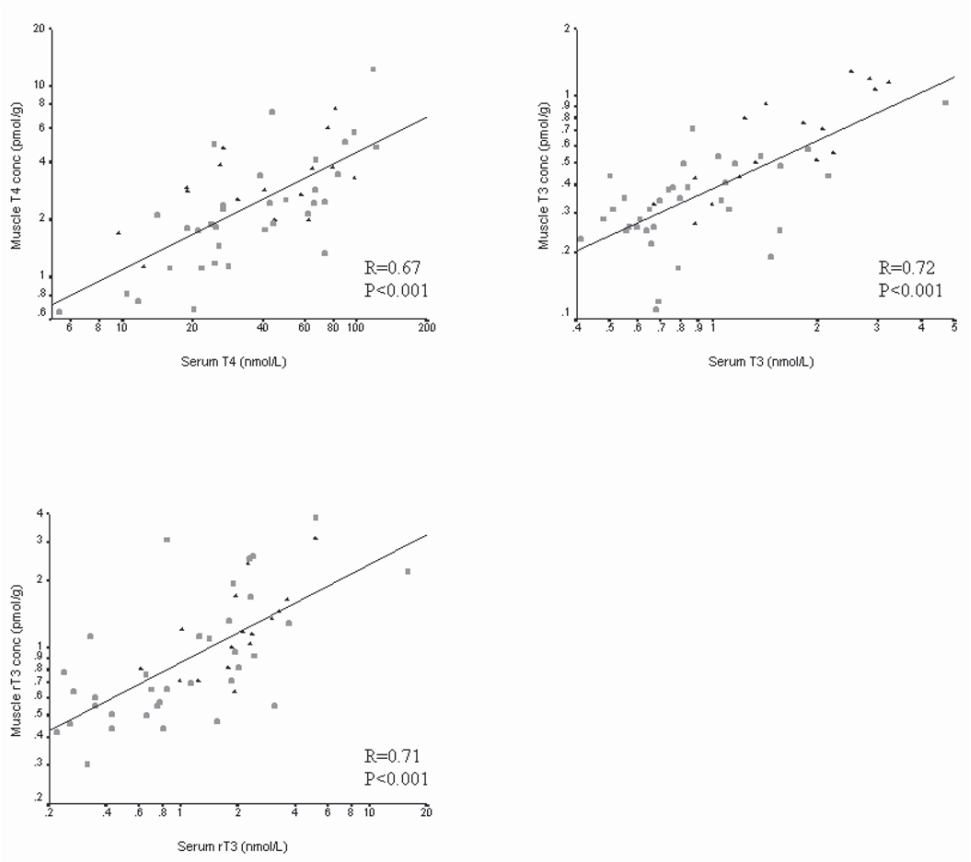


**Figure 2.** Correlation analysis of serum T4, T3, and rT3 levels with local T4, T3, and rT3 concentrations in skeletal muscle. Patients who did not receive thyroid hormone treatment are represented by ● , whereas patients who received thyroid hormone therapy are represented by ▲ . R represents Spearman's correlation coefficient. To convert values for serum T4 to µg/dL, multiply by 0.0777, and to convert values for serum T3 and rT3 to ng/dL, multiply by 64.9. Muscle concentrations of T4, T3, and rT3 are shown in picomoles per gram wet weight. To convert values for muscle T4 to nanograms per gram, divide by 1.287; to convert values for muscle T3 and rT3 to nanograms per gram, divide by 1.54.

**Table 5.** Correlation analysis of tissue (liver or skeletal muscle) iodothyronine levels and tissue deiodinase activities.

| | TH treatment | | Tissue T4 (ng/g) | T3 (ng/g) | rT3 (ng/g) | T3/ T4 | T3/rT3 |
|---|---|---|---|---|---|---|---|
| Liver D1 (fmol/mg/min) | Yes/No (N=64) | R | -0.13 | 0.10 | -0.54 | 0.08 | 0.57 |
| | | P | 0.92 | 0.42 | <0.001 | 0.56 | <0.001 |
| | No (N=43) | R | -0.09 | 0.28 | -0.64 | 0.27 | 0.73 |
| | | P | 0.57 | 0.07 | <0.001 | 0.08 | <0.001 |
| Liver D3 (pmol/mg/min) | Yes/No (N=64) | R | 0.07 | 0.09 | 0.28 | -0.03 | -0.20 |
| | | P | 0.57 | 0.50 | 0.03 | 0.82 | 0.11 |
| | No (N=43) | R | 0.11 | 0.09 | 0.31 | -0.08 | -0.21 |
| | | P | 0.47 | 0.57 | 0.05 | 0.60 | 0.17 |
| Muscle D3 (pmol/mg/min) | Yes/No (N=62) | R | 0.11[a] | -0.06 | 0.57 | -0.15[a] | -0.53[a] |
| | | P | 0.38 | 0.66 | <0.001 | 0.24 | <0.001 |
| | No (N=42) | R | -0.02[a] | -0.28 | 0.48 | -0.19[a] | -0.64[a] |
| | | P | 0.91 | 0.07 | 0.001 | 0.24 | <0.001 |

R represents Spearman's Correlation coefficient, [a] N = 61, and 41 respectively

In this study, liver D1 activity showed a strong negative correlation with liver rT3 levels (R=-0.54, P<0.001) and a positive correlation with the liverT3/rT3 ratio (R=0.57, P<0.001), but no relation with liver T4 or T3 levels (Table 5, Figure 3). After exclusion of all patients who were treated with thyroid hormone, also liver T3 levels tended to be higher in patients with higher liver D1 activity (R=0.28, P=0.07). D3 activity in liver was positively correlated with the liver rT3 concentration (R=0.28, P=0.03), and D3 activity in skeletal muscle showed a significant correlation with both skeletal muscle rT3 (R=0.57, P<0.001) and the T3/rT3 ratio (R=-0.53, P<0.001) (Table 5, Figure 3). In patients who did not receive thyroid hormone therapy, skeletal muscle T3 levels tended to be lower in patients with a higher muscle D3 activity (R=-0.28, P=0.07).

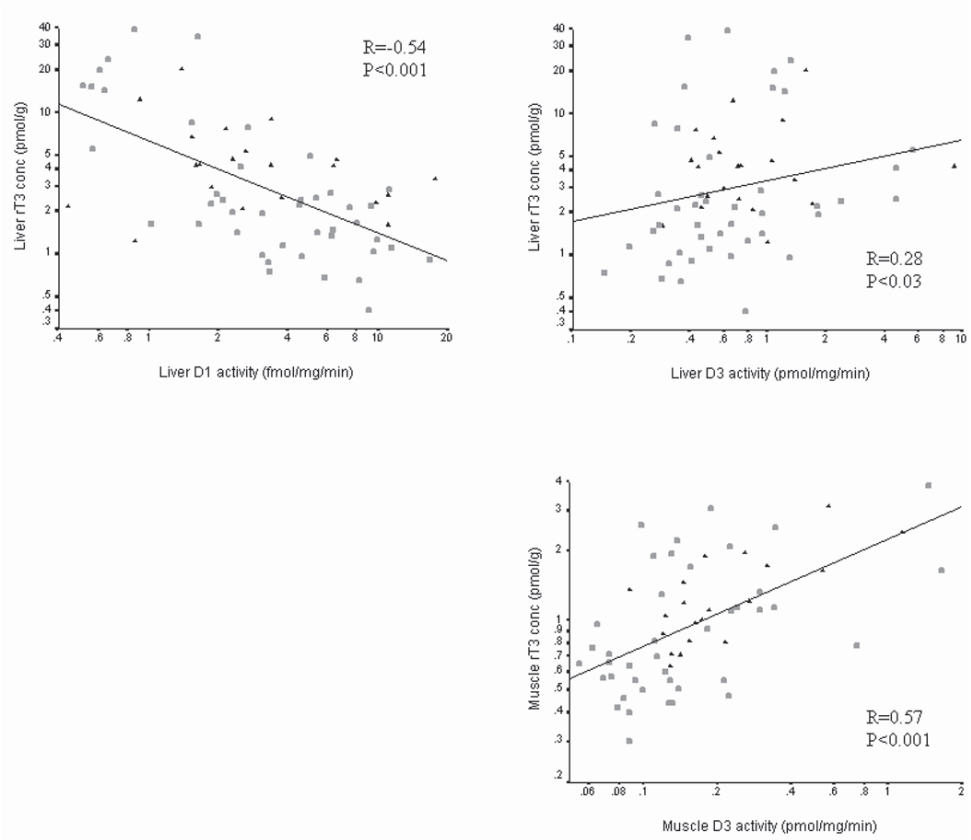


**Figure 3.** Correlation analysis of liver D1, liver D3, and muscle D3 activity with local rT3 concentrations in liver or skeletal muscle. Patients who did not receive thyroid hormone treatment are represented by ●, whereas patients who received thyroid hormone therapy are represented by ▲. R represents Spearman's correlation coefficient. Tissue concentrations of T4, T3, and rT3 are shown in picomoles per gram wet weight. To convert values for tissue T4 to nanograms per gram, divide by 1.287; to convert values for tissue T3 and rT3 to nanograms per gram, divide by 1.54.

## *MCT8 expression and different thyroid parameters*

MCT8 expression in liver showed a positive correlation with liver D1 activity (R=0.34, P=0.01) and a negative correlation with liver D3 activity (R=-0.52, P<0.001) (Table 6, Figure 4). MCT8 expression in skeletal muscle showed a positive relation with muscle D3 activity (R=0.32, P=0.006) (Table 6, Figure 4).

Liver MCT8 expression was negatively correlated with serum and liver rT3 concentrations (P<0.001 and P=0.004) and positively with the serum and liver T3/rT3 ratio (P<0.001 and P= 0.003), but not with the other iodothyronines. Multiple

linear regression analysis showed that the relation of liver MCT8 expression and liver iodothyronine levels was due to the positive relation of liver MCT8 expression with liver D1 activity, since the relation of MCT8 on liver rT3 and the T3/rT3 ratio disappeared completely when liver D1 was added to the regression model (P=0.11 and P=0.40). However, after adjustment for liver D1, the relation of liver MCT8 with serum rT3 and T3/rT3 remained significant (P=0.02 and P=0.02). Muscle MCT8 expression was positively correlated with muscle rT3 concentrations and negatively with the muscle T3/rT3 ratio (P=0.01 and P=0.03), which was due to the positive relation of muscle MCT8 expression and muscle D3 activity (P=0.34 and P=0.33, after addition of muscle D3 to the regression model).

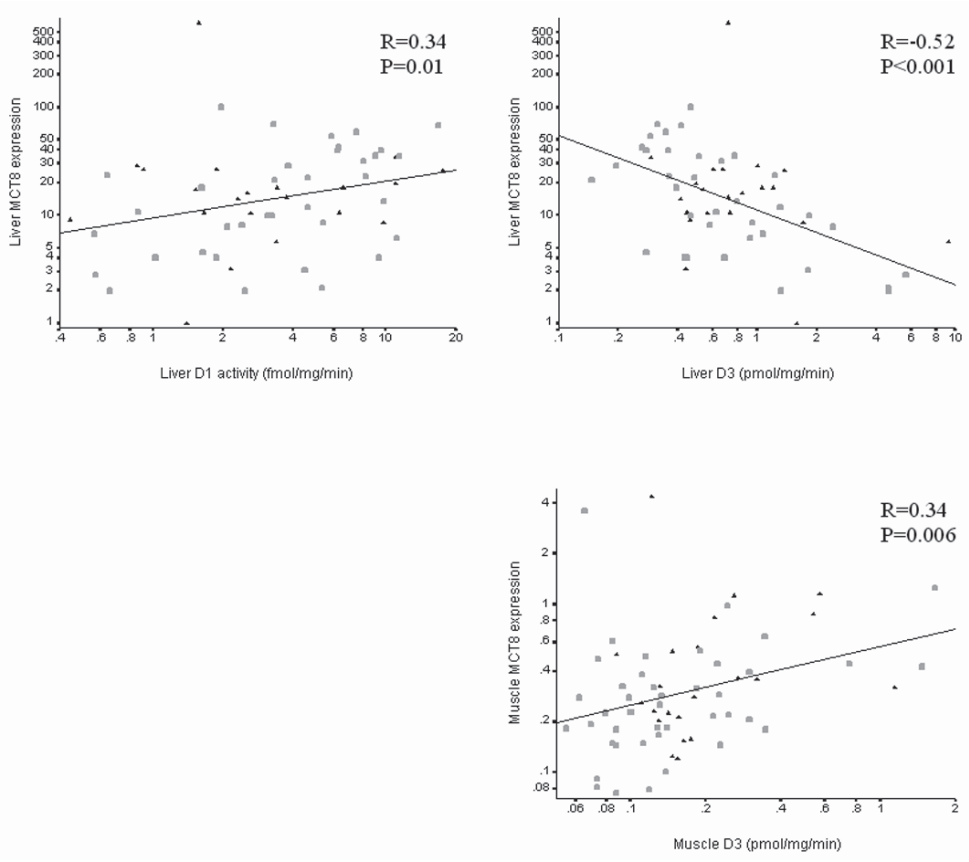


**Figure 4.** Correlation analysis of liver D1and liver D3 activity with liver MCT8 expression, and of muscle D3 activity with muscle MCT8 expression. MCT8 mRNA levels are expressed relative to the house keeping gene GAPDH (see materials and methods). Patients who did not receive thyroid hormone treatment are represented by ●, whereas patients who received thyroid hormone therapy are represented by ▲. R represents Spearman's correlation coefficient.

**Table 6.** Correlation analysis of liver or skeletal muscle MCT8 expression, the serum / tissue ratio of the different iodothyronines, and tissue deiodinase activities

| | TH treatment | | Liver MCT8 expression | TH treatment | | Muscle MCT8 expression |
|---|---|---|---|---|---|---|
| Serum T4 / Tissue T4 ratio | Yes/No (N=42) | R<br>P | 0.28<br>0.07 | Yes/No (N=49) | R<br>P | -0.27<br>0.07 |
| | No (N=27) | R<br>P | 0.30<br>0.13 | No (N=33) | R<br>P | -0.17<br>0.35 |
| Serum T3 / Tissue T3 ratio | Yes/No (N=42) | R<br>P | 0.15<br>0.35 | Yes/No (N=50) | R<br>P | 0.17<br>0.23 |
| | No (N=27) | R<br>P | 0.15<br>0.45 | No (N=34) | R<br>P | 0.16<br>0.36 |
| Serum rT3 / Tissue rT3 ratio | Yes/No (N=42) | R<br>P | -0.17<br>0.27 | Yes/No (N=50) | R<br>P | -0.16<br>0.27 |
| | No (N=27) | R<br>P | -0.34<br>0.08 | No (N=34) | R<br>P | -0.23<br>0.19 |
| Tissue D1 (fmol/mg/min) | Yes/No (N=57) | R<br>P | 0.34<br>0.01 | | | |
| | No (N=36) | R<br>P | 0.44<br>0.007 | | | |
| Tissue D3 (pmol/mg/min) | Yes/No (N=57) | R<br>P | -0.52<br><0.001 | Yes/No (N=64) | R<br>P | 0.34<br>0.006 |
| | No (N=36) | R<br>P | -0.63<br><0.001 | No (N=42) | R<br>P | 0.29<br>0.06 |

R represents Spearman's Correlation coefficient

If MCT8 expression would play an important role in transmembrane transport of iodothyronines, an effect of MCT8 expression on the ratio of the serum over tissue concentrations of the different iodothyronines would be expected. However, neither in liver, nor in skeletal muscle, was MCT8 expression related to the ratio of the serum over tissue concentration of the different iodothyronines (Table 6).

## Discussion

Plasma T3 decreases and plasma rT3 increases during critical illness, and the magnitude of these changes is related to the severity of the disease (2-5). Although intensive insulin therapy has been shown to result in a decreased morbidity and mortality in intensive care patients (16, 21), no differences in liver and skeletal muscle iodothyronine levels were found between the two insulin treatment groups. This is in line with previous studies, in which we found no effect of insulin treatment on serum thyroid parameters and/or deiodinase activities (13, 22). It should be noted, however, that all patients in this study died in the ICU. Since insulin therapy has a beneficial effect on survival, the possibility of a selection bias (sicker patients in the group receiving intensive insulin therapy), cannot be excluded.

Whether the reduction in serum T3 is a maladaptation that should be treated, or whether it is a beneficial adaptation, is still a controversial issue (2, 8, 9). A positive effect on clinical outcome of thyroid hormone substitution during critical illness has so far not been demonstrated (10-12). When patients are treated with T4 or T3, it is unclear if thyroid hormone is taken up by tissues and metabolized. In this study, patients were treated with a combination of T4 and T3, if they had a serum T4 concentration below 50 nmol/l and concomitantly strictly defined clinical symptoms suggestive of hypothyroidism (13). Patients who received glucocorticoids, which are known to inhibit TSH secretion, received thyroid hormone substitution more often. It cannot be ascertained to what extent the requirement for thyroid hormone replacement is caused by administration of the glucocorticoids or by the underlying illness. Due to the lack of a good control group, no conclusions about the possible beneficial or negative effects of thyroid hormone substitution can be drawn from this study. It can be concluded, however, that the higher serum T3 levels, although still in the low normal range, in patients who received thyroid hormone substitution therapy were accompanied by higher levels of T3 in liver and skeletal muscle, with a 2-fold greater increase in liver T3 than in serum T3. These data indicate different effects of thyroid hormone treatment on T3 levels in different tissues, which is in line with studies in thyroidectomized rats, demonstrating tissue-specific regulation of tissue thyroid hormone concentrations (23-25). In these studies it was also shown that neither substitution with T4, nor with T3, was able to restore T3 in plasma and all tissues of these thyroidectomized rats to similar levels as in euthyroid rats (23-25).

A major disadvantage of substitution with thyroid hormone itself, is that the hypothalamus-pituitary-thyroid axis is bypassed. This may result in over-treatment and TSH suppression, whereas an increase in serum TSH marks the onset of recovery and concomitantly drives the increase in serum T4 (2, 26, 27). If a combina-

tion of T4 and T3 is given, the local regulation of thyroid hormone bioactivity by T4 to T3 conversion is also bypassed, which may be an argument against giving a combination of T4 and T3. On the other hand, a randomized prospective study in hypothyroxinemic critically ill patients showed an increase in serum T4, but not in serum T3, after T4 treatment (10), which can be explained by the decreased D1 and D2 activity during critical illness (13) (22). It is unclear from our study if substitution therapy with T4 alone would have resulted in higher levels of serum and/or tissue T3. Intervention with hypothalamic releasing factors, which restores pulsatile pituitary hormone secretion, normalizes peripheral hormone levels, and keeps the negative feedback loop intact, might be a more successful approach (28-30).

Serum iodothyronine levels showed a strong positive correlation with both liver and skeletal muscle iodothyronine levels. It should be noted, that we do not have any data on the free concentrations of T4 and T3 in the sera and tissues of these patients, nor do we have data on the (intracellular) localization of thyroid hormone. However, liver T4 and tissue T3 in general are predominantly located intracellularly, in contrast to muscle T4, which is predominantly located in plasma and interstitial fluids (31, 32). Our data indicate that the decrease in serum T4 and T3 levels during critical illness also results in decreased levels of T4 and T3 in liver and skeletal muscle. The decreased expression of liver D1 during critical illness supports this (1, 13), since D1 is a very sensitive marker of tissue thyroid hormone status (33). An autopsy study, in which 12 patients who died of severe nonthyroidal illnesses were compared with 10 patients who died acutely from trauma, showed significantly decreased levels of T4 and T3 in liver, but not in skeletal muscle, of the severely ill patients (7). In our study however, the relation between serum and tissue iodothyronine levels was weakest for liver T4, compared to the other iodothyronines in liver and muscle. This might suggest that other factors than serum concentrations, such as deiodination and regulation of transport, are more important in the regulation of liver T4 concentration than in the regulation of liver T3 or muscle iodothyronine concentration.

A low D1 activity results in a reduced T3 production and rT3 clearance (5). The negative correlation of liver D1 activity with the local rT3 concentration in liver, and the positive correlation with the liver T3/rT3 ratio, are in line with this. An induction of D3 activity enhances the clearance of T3 and the production of rT3 (5, 13). The positive correlation of tissue D3 activity with rT3 levels in both liver and skeletal muscle, and the negative correlation of muscle D3 with the local T3/rT3 ratio are also in agreement with this. Furthermore, in patients who did not receive thyroid hormone substitution, liver D1 activity showed a trend with higher

levels of liver T3, whereas muscle D3 activity showed a trend with lower levels of muscle T3. The complete absence of muscle D2 activity in these patients is remarkable and must have resulted in a decreased local T3 production, since D2 activity is present in skeletal muscle of normal subjects (5, 34, 35). A possible explanation for the lack of D2 activity is the short half-life of functional D2 protein. D2 has an approximately 45-min half-life in euthyroid conditions due to selective ubiquitin-mediated endoplasmatic reticulum-associated degradation (36). This short half-life may cause rapid post-mortem D2 inactivation. However, since muscle biopsies were taken within 45 min after death, post-mortem decay of D2 can not solely explain the lack of D2 activity in skeletal muscle samples of these patients. The shortest interval between entry in the ICU and isolation of tissue samples was between 24 and 48 h. Thus, in view of the short half-life of the D2 protein, it may well have disappeared from skeletal muscle if its expression is acutely suppressed in severe illness. These data indicate an important role during critical illness for liver D1 and D3, and skeletal muscle D2 and D3 activity in the regulation of local concentrations of thyroid hormone, and thus of local thyroid hormone bio-activity.

In addition to serum iodothyronine levels and tissue deiodinase expression, transmembrane transport of iodothyronines is also important in the regulation of thyroid hormone bioactivity (14). Uptake of T4 by human hepatocytes is temperature, Na-, and energy dependent (37), and kinetic analyses indicate that T4 and T3 cross the plasma membrane by different transporters (38, 39). Recently, human MCT8 was identified as the first known thyroid hormone specific transporter, with a preference forT3 over T4 in humans (unpublished data). Although MCT8 transport activity is not Na- and/or energy dependent, MCT8 is expressed in, among other tissues, liver and skeletal muscle (15). An effect of MCT8 expression on the ratio of the serum over tissue concentration of the iodothyronines would be expected, if MCT8 expression is important in transmembrane transport of iodothyronines in a certain tissue. In this study, neither liver, nor skeletal muscle MCT8 expression were related to the ratio of the serum over tissue concentration of T4, T3, or rT3, suggesting that MCT8 is not crucial in the transport of these iodothyronines over the plasma membrane in liver and skeletal muscle. This is supported by the fact that inactivating mutations in MCT8 result in elevated levels of T3, low levels of rT3, and in a phenotype of severe mental retardation, but that there is currently no evidence for liver or muscle hypothyroidism in these patients (40). Alternatively, MCT8 may facilitate both in- and efflux, without having a net effect on intracellular iodothyronine levels in liver and skeletal muscle. A relation between MCT8 activity

and the tissue vs. serum ratio of the different iodothyronines can obviously not be excluded from these data, since we only looked at mRNA expression of MCT8.

Although our data suggest that MCT8 expression does not play a major role in iodothyronine uptake in liver and skeletal muscle, there was a close relation between MCT8 expression and deiodinase activities. Liver D1 activity was positively associated with liver MCT8 expression, whereas liver D3 activity showed a negative relation. This relation was opposite in skeletal muscle, since muscle D3 activity was positively correlated with muscle MCT8 expression. This might suggest a different role for these tissues during critical illness. The liver plays a major role in serum T3 production (5), and the decreased T3 production during critical illness may not only be explained by a decreased D1 activity, but also by a decreased transport of T4 into D1 containing tissues (14). Muscle D2 activity, which is present in skeletal muscle of normal subjects (5, 34, 35), was completely absent in these patients, whereas muscle D3 activity was induced. The positive relation between muscle D3 activity and muscle MCT8 expression suggests an increased uptake of iodothyronines in skeletal muscle under conditions when D3 activity is high. This might result in an increased substrate availability for muscle D3, suggesting that during critical illness skeletal muscle is more important for the inactivation than for the activation of thyroid hormone. However, this is highly speculative, especially since our data also suggest that other transporters than MCT8 are more important in the regulation of thyroid hormone uptake in liver and skeletal muscle.

In conclusion, this is the first study on the relationship between serum and tissue iodothyronine levels in a large group of critically ill patients. Serum iodothyronine levels were positively correlated with both liver and muscle iodothyronine levels, indicating that the decrease in serum T4 and T3 levels during critical illness also results in decreased levels of tissue T4 and T3. Higher serum T3 levels in patients who received thyroid hormone therapy were accompanied by higher levels of liver T3 and rT3, and by higher levels of muscle T4, T3, and rT3, indicating tissue specific effects of thyroid hormone treatment. Furthermore, tissue rT3 and T3/rT3 were correlated with tissue deiodinase activities, whereas MCT8 expression was not related to the ratios of the serum over tissue concentrations of the different iodothyronines.

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# *Chapter B5*

## Expression of the glucocorticoid receptor variants α, β, and P during critical illness

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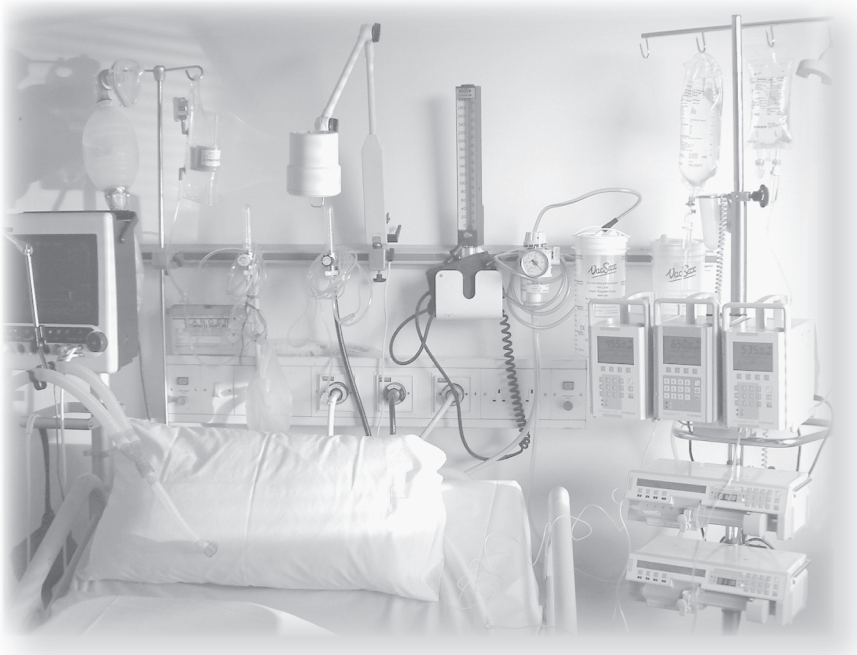

## Abstract

**Introduction**: Critical illness results in an activation of the hypothalamic-pituitary-adrenal-axis (HPA), which might be accompanied by a peripheral adaptation in glucocorticoid sensitivity. Tissue sensitivity is determined by the active glucocorticoid receptor (GRα), of which two splice variants involving the hormone binding domain exist (GRβ and GR-P).

**Methods**: We assessed mRNA expression of the GRα, GRβ and GR-P splice variants in liver (n=58) and skeletal muscle (n=65) samples of patients who had died after intensive care, and who had been randomized for insulin treatment. We also analyzed if expression of the GR splice variants was associated with severity of illness, cortisol levels, and treatment.

**Results**: GRα and GR-P mRNA constituted 87±8% and 13±2% of total GR mRNA in liver, whereas GRβ mRNA could only be amplified in five liver samples. All variants were present in muscle (α=96±11%, P=3.9±0.4%, β= 0.010±0.002%). GR expression was not different between patients treated with intensive or conventional insulin therapy. A strong positive linear relation was observed between the expression of the different GR variants, both in liver and in skeletal muscle (P<0.001 for all). Different indicators of severity of illness showed a negative relation with GR expression, but not with the α/P or α/β ratio. Serum peri-mortem cortisol levels were negatively associated with liver GRα and muscle GR-P expression. Both liver GRα and GR-P expression, but not muscle GR expression, were substantially lower in patients who received exogenous glucocorticoids (P<0.01).

**Conclusion**: The negative relation of mRNA levels of the receptor variants with circulating cortisol and severity of illness suggests a down-regulating effect of HPA-activation on GR expression. In contrast to muscle GR expression, liver GR expression was substantially lower in patients receiving exogenous glucocorticoids.

## Introduction

Critical illness is accompanied by activation of the hypothalamic-pituitary-adrenal axis (HPA-axis), resulting in increased serum cortisol concentrations (1-5). During critical illness, the HPA-axis undergoes a biphasic change. In the acute phase, increased cortisol production appears to be induced by increased levels of ACTH (6-8). In this acute phase, cortisol induces shifts in protein and carbohydrate metabolism. It enhances hepatic glucose output by stimulating gluconeogenesis, whereas in muscle glucose uptake, glucose utilization, and protein synthesis are inhibited, the latter resulting in an increased amino acid availability for gluconeogenesis. This

results in instantly available energy, whereas anabolism is postponed. In the more chronic phase, ACTH levels are low and the sustained cortisol production is likely to be driven by pro-inflammatory mediators like IL-6 (9-11). The possible benefit of hypercortisolism in prolonged critical illness is questionable, since it may theoretically result in immune suppression, impaired healing, myopathy and insulin resistance.

Next to the availability of cortisol, the sensitivity of target tissues is important in the regulation of cortisol bioactivity. Cortisol exerts its effects through intracellular receptors, which either directly bind to DNA or interact with other transcription factors (12). In addition to the active glucocorticoid receptor (GR) protein (GRα), two splice variants involving the hormone binding domain exist (GRβ and GR-P). GRα uses exons 2-8 and part of exon 9α as a coding region (777 amino acids), whereas GRβ uses part of exon 9β instead of 9α, which renders GRβ unable to bind glucocorticoids (742 amino acids) (13, 14). The third variant, GR-P, is a truncated protein (only 676 amino acids) that differs from the GRα and GRβ, because it is encoded by the exons 2-7 and part of intron 7, whereas exons 8 and 9 are missing (15, 16). The GRβ variant has been reported to have a dominant negative effect on GRα (13, 17), whereas expression of the GR-P variant increases the activity of GRα in several transiently transfected cell lines (16). So far, no data are available about the expression of these GR splice variants during critical illness.

In a recent study involving 1548 intensive care unit (ICU) patients, it was shown that maintaining normoglycemia during critical illness, using intensive insulin treatment, reduced ICU mortality by 43% (18). Furthermore, morbidity was reduced resulting in a decreased need for prolonged mechanical ventilation and intensive care. There is a close interaction between cortisol and carbohydrate metabolism, and since treatment with glucocorticoids and insulin have both been shown to affect outcome of ICU patients (18, 19), we hypothesized that part of the beneficial effects of intensive insulin treatment might be explained by direct or indirect effects on cortisol bioactivity.

We therefore assessed mRNA expression of the GRα, GRβ and GR-P splice variants in liver and skeletal muscle samples of patients who had died after intensive care, and who had been randomized for insulin treatment. We also analyzed if the expression of the GR splice variants was associated with severity of illness, cortisol levels, and other types of treatment.

## Patients and Methods

### *Patients and concomitant treatment*

This study was part of a large randomized controlled study on intensive insulin treatment in ICU patients (N=1548), of which the major clinical outcomes have been published in detail elsewhere (18). All mechanically ventilated adult patients were eligible for inclusion in this trial after informed consent from the closest family member. On admission, patients were randomly assigned to either strict normalization of blood glucose (80-110 mg/dl) with intensive insulin therapy or the conventional approach, in which insulin infusion is initiated only when blood glucose exceeds 215 mg/dl, and which resulted in an average blood glucose of 150-160 mg/dl. The study protocol was approved by the Ethical Review Board of the University of Leuven School of Medicine before start of the study and patients were included after informed consent from the closest family member.

For the current analysis a total of 79 patients, out of the 98 patients who died in the original study, were included. Eleven out of 79 patients had died of a cardiovascular collapse, 36 of multiple organ failure (MOF) with sepsis, 26 of MOF with systemic inflammatory response syndrome, and 6 patients of severe brain damage. Blood samples were obtained from 63 patients, liver biopsies from 58 patients, and skeletal muscle (rectus abdominis) biopsies from 65 patients within minutes after death (25.2 ± 20.0 (SD), range [5-97 min] for liver and 20.7 ± 19.7, range [0-95 min] for skeletal muscle). There was no relation between mRNA expression and post-mortem time of the samples in this study. From 41 patients blood, liver as well as skeletal muscle samples were available. 42 patients of whom a liver sample was available, and 48 patients of whom a muscle sample was available, stayed on the ICU for more than 5 days. Of those patients, also a serum sample obtained at 6.00 am on the last day was available.

Patients were treated with hydrocortisone if they had clinical signs of relative adrenal failure and, concomitantly, a baseline cortisol of < 15 μg/dl and a response to 250 μg ACTH of < 7μg/dl. Methylprednisolone was given to treat acute respiratory distress syndrome (ARDS) and/or to achieve suppression of the inflammatory cascade. All patients included in this study had died in the ICU, and the cause of death was determined both clinically by the attending ICU physician and by post-mortem examination. The pathologist was unaware of insulin treatment allocation. Relevant patient characteristics are summarized in Table 1.

**Table 1.** Descriptive statistics of the population analyzed in this study

| | Conventional Insulin treatment | Intensive Insulin treatment | *P* |
|---|---|---|---|
| No. of patients | 54 | 25 | |
| Age (years) | 68.6 ± 12.1 | 68.5 ± 12.4 | 0.68 |
| BMI (kg/m2) | 24.9 ± 3.4 | 26.4 ± 6.6 | 0.46 |
| ICU stay (days) | 16 (7-36) | 11 (4-25) | 0.25 |
| No. of male patients (%) | 37 (69%) | 18 (72%) | 0.80 |
| No. of patients receiving GC treatment (%) | 28 (52%) | 14 (56%) | 0.81 |
| No. of patients receiving GC treatment on LD (%) | 17 (31%) | 9 (25%) | 0.80 |
| APACHE II score Day 1 | 12 (10-19) | 16 (10-18) | 0.53 |
| APACHE II score Last Day | 20 (16-28)[a] | 21 (15-28)[b] | 0.81 |
| TISS-28 score Day 1 | 42 (35-48) | 39 (32-48) | 0.24 |
| TISS-28 score Last Day | 39 (34-43) | 40 (35-47) | 0.34 |

[a] N= 37, [b] N= 17
Age and BMI are shown as mean ± SD, whereas ICU stay, and the APACHE II and TISS-28 scores are shown as median (IQR). P values represent Mann-Whitney U tests.
GC, glucocorticoids; LD, Last Day; IQR, Interquartile range

Severity of illness was scored using the Acute Physiology and Chronic Health Evaluation (APACHE II) score (20) and the simplified Therapeutic Intervention Scoring System (TISS-28) (21, 22). Higher scores indicate more severe illness and a higher number of therapeutic interventions, respectively.

### *Serum analyses*

Serum cortisol levels were measured in serum that was obtained peri-mortem and in serum that was obtained at 6.00 am on the day the patient died using chemoluminescence assays on an Immulite 2000 (D.P.C., Los Angeles, CA). Within assay coefficient of variation amounted to 5.55%. Cross-reactivity of the cortisol assay with methylprednisolone is 21-23 %, and cortisol levels were not adjusted for this cross-reactivity in patients who received methylprednisolone treatment. Unfortunately, not enough peri-mortem serum was available for CBG measurements. Serum IGFBP-I concentrations were measured using an in-house enzyme-linked immunoassay specific for human IGFBP-1 (23).

### *RNA isolation and reverse transcription*

RNA was isolated from liver samples using the High Pure RNA Tissue Kit (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's protocol. With this method, RNA is bound to a glass fiber fleece, and after DNA digestion with DNAse I and several washing steps, eluted with distilled water. RNA isolation from skeletal muscle failed, because skeletal muscle tissue obstructed the fleece. Therefore, skeletal muscle RNA was isolated using Trizol reagent (GIBCO BRL, Breda, The Netherlands) as an initial isolation step, with a further purification of the RNA using the High Pure RNA Tissue Kit. RNA concentrations were determined using the RiboGreen® RNA Quantitation Kit (Molecular Probes, Leiden, The Netherlands). All samples were diluted to 0.1 µg/µl, and 1 µg was used for cDNA synthesis using the TaqMan™ Reverse Transcription Kit (Roche Diagnostics).

### *Real-time RT- PCR*

GRα, GRβ, and GR-P mRNA levels were determined in all liver and skeletal muscle samples using the qPCR-core kit (Eurogentec, Liege, Belgium). The ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands) was used, which employs TaqMan™ chemistry for highly accurate quantitation of mRNA levels. In order to minimize differences between the separate real-time RT-PCR assays for each of the three splice variants we designed assays using one common forward primer, one common probe and a unique reverse primer for each of the splice variants. We also aimed at amplicons of the same size. This resulted in amplicons that were about twice the size usually employed, which showed acceptable Ct values and also resulted in similar efficiencies (as tested with plasmids containing the three splice variants). FRET- probes and primers were obtained from Biosource (Nivelles, Belgium), sequences and concentrations are given in Table 2. GR mRNA levels were calculated as absolute values and relative to those of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene measured in a parallel separate assay. To exclude the possibility of bias caused by this housekeeping gene, all data were also calculated relative to the hypoxanthine phosphoribosyltransferase (HPRT) gene. The GAPDH probe and primers were provided as a pre-optimized control system (Applied Biosystems), the HPRT gene probes and primers are given in Table 2. Unless mentioned specifically, the choice of the housekeeping gene did not influence interpretation of the data.

**Table 2.** Sequence and concentration of primers and probes that were used for determination of mRNA levels by quantitative real-time PCR

| | Sequence | Concentration |
|---|---|---|
| GR Taq Man assay | | |
| GR-fwd | 5'-TGTTTTGCTCCTGATCTGA-3' | 300 nmol/L |
| GR-probe | 5'-FAM-TGACTCTACCCTGCATGTACGAC-TAMRA-3' | 200 nmol/L |
| GR-α-rev | 5'-TCGGGGAATTCAATACTCA-3' | 300 nmol/L |
| GR-P-rev | 5'-GTTTCTGCCATACCTATTTG-3' | 300 nmol/L |
| GR-β-rev | 5'-TGAGCGCCAAGATTGT-3' | 300 nmol/L |
| HPRT TaqMan assay | | |
| HPRT-fwd | 5'-CACTGGCAAAACAATGCAGACT-3' | 500 nmol/L |
| HPRT-probe | 5'-FAM-CAAGCTTGCGACCTTGACCATCTTTGGA-TAMRA-3' | 200 nmol/L |
| HPRT-rev | 5'-GTCTGGCTTATATCCAACACTTCGT-3' | 500 nmol/L |

The real-time PCR protocol was 2 min at 50 C, 10 min at 95 C, followed by 40 cycles of 15 s at 95 C and 1 min at 60 C. According to the manufacturer's guidelines, data were expressed as Ct values, which represent the cycle number at which probe-derived dye fluorescence reaches the calculated threshold value. Data expressed relative to the housekeeping gene are shown as 100 x ($2^{\Delta Ct}$). ΔCt is the Ct value of the housekeeping gene minus the Ct value of the target gene.

### *Immunoblotting*

Liver and skeletal muscle samples were homogenized in PE buffer and 50 μg protein of each sample was separated on 10% SDS-PAGE gels in the Mini-Protean III system (Bio-Rad) according to manufacturer's instructions. After electrophoresis, the proteins were blotted to nitrocellulose membrane (Hybond ECL; Amersham-Pharmacia Biotech, Germany), incubated with primary antiserum (GR N-terminal antibody PA1-511A, Affinity BioReagents, USA, dilution 1: 500), and subsequently incubated with peroxidase-conjugated secondary antibody as described previously (24). Blots were developed using the Western Lightning Chemiluminescence Plus according to the manufacturers instructions (Perkin Elmer, Zaventem, Belgium) and exposed to film (Amersham).

COS cell homogenates transfected with the previously described pRShGRα plasmid were used as a positive control (25).

### *Statistical analysis*

Data were analyzed using the statistical program SPSS 10.0.7 for Windows (SPSS, Chicago, IL). Logarithmic transformations were applied in order to normalize variables and to minimize the influence of outliers, when appropriate. All analyses were done on the whole group, as well as on subgroups treated or not treated with glucocorticoids. Data were analyzed using one-way ANOVA tests, with a post-hoc Fisher's least significant difference (LSD) test for multiple comparisons, Student's t-tests, Mann-Whitney U tests, Kruskal-Wallis H tests, and linear regression analyses, when appropriate. Data are shown as median (interquartile range (IQR)) in case of a non-normal distribution, and as mean ± SD in case of a normal distribution. Correlation coefficients were obtained by Spearman's correlation analysis. One liver sample and one skeletal muscle sample were excluded from the analysis, because both GRα and GR-P mRNA, adjusted for GAPDH, were higher than the mean + 3SDs. Unless stated otherwise, all GR mRNA levels shown are expressed relative to the GAPDH housekeeping gene.

## Results

### *Tissue expression of the GR variants*

GRα and GR-P mRNA were observed in liver (α=87 ± 8%, P=13 ± 2% of total GR mRNA). GRβ mRNA could only be amplified in five liver samples. In those five samples, on average 8 more cycles were needed than for GRα before the signal became higher than the calculated threshold value, indicating that the expression of GRβ mRNA in these patients was 256 times lower than GRα. A strong, positive relation was observed between GRα and GR-P mRNA expression (R=0.67, P<0.001) (Fig. 1A).

All three variants were present in skeletal muscle (α=96 ± 11%, P=3.9 ± 0.4%, β= 0.010 ± 0.002% of total GR mRNA). A strong, positive relation was observed between GRα and GR-P (R=0.86, P<0.001) and GRα and GRβ (R=0.66, P<0.001) in muscle (Fig. 1B and 1C). GRα mRNA levels per μg RNA were ~10 times higher in skeletal muscle than in liver. The α/P ratio was significantly higher in muscle tissue than in liver tissue (9.5 ± 0.8 vs. 25.4 ± 1.2, P<0.001). No relation was observed between the α/P ratios of liver vs muscle (P=0.59). The α/β ratio in skeletal muscle was 10330 ± 1082.

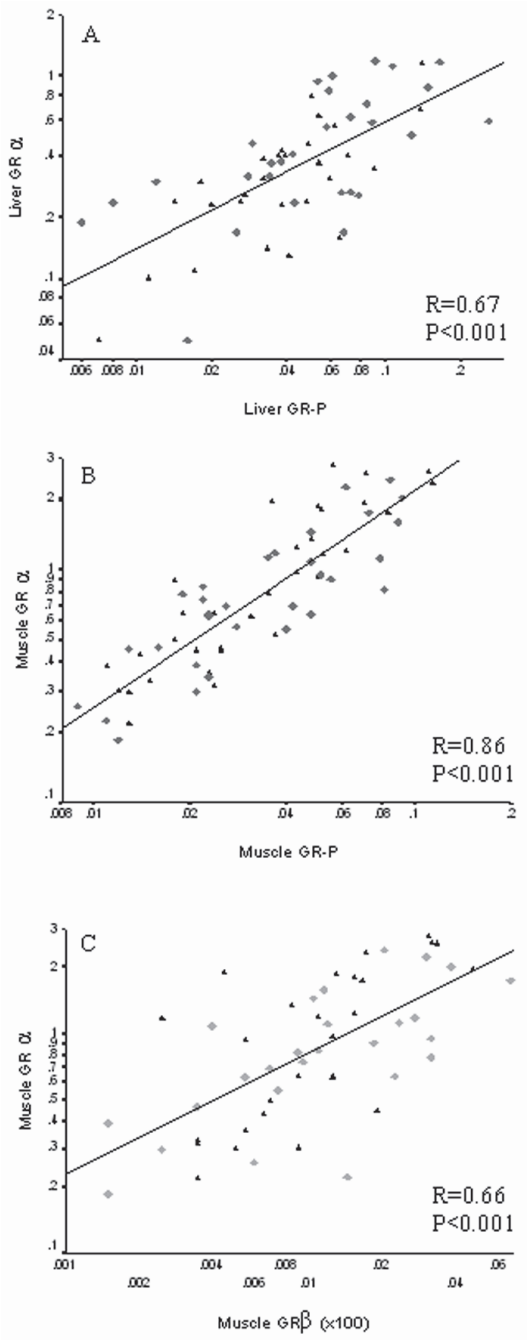


**Figure 1**. Correlation analysis of liver GRα and GR-P (A), of skeletal muscle GRα and GR-P (B), and of skeletal muscle GRα and GR-β expression (C). Patients who did not receive glucocorticoid treatment are represented by ●, whereas patients who received glucocorticoid therapy are represented by ▲. R represents Spearman's correlation coefficient.

### *Cortisol levels, GR expression and insulin treatment*

Because intensive insulin treatment reduced ICU mortality by 43%, less tissue samples were available from patients in this group. The mRNA expression of the different GR variants, or their ratios, were not different between patients treated with conventional or intensive insulin therapy, neither in liver nor in skeletal muscle (Table 3). Nor was there any difference in peri-mortem serum cortisol levels between patients treated with conventional or intensive insulin therapy (Table 3). Analysis of the subgroup of patients who were not treated with glucocorticoids gave similar results.

**Table 3.** GR expression and cortisol levels in patients treated with conventional or intensive insulin treatment

| | Conventional Insulin Treatment | Intensive Insulin Treatment | *P* |
|---|---|---|---|
| Liver GRα | 0.45 ± 0.05 | 0.43 ± 0.07 | 0.96 |
| Liver GR-P | 0.054 ± 0.007 | 0.065 ± 0.01 | 0.86 |
| Liver GRα/P | 10.40 ± 0.96 | 8.18 ± 1.35 | 0.28 |
| Muscle GRα | 0.92 ± 0.11 | 1.12 ± 0.15 | 0.45 |
| Muscle GR-P | 0.040 ± 0.004 | 0.041 ± 0.006 | 0.89 |
| Muscle GRα/P | 23.89 ± 1.43 | 28.39 ± 1.97 | 0.29 |
| Serum Cortisol (nmol/L) | 697 (460-1084) | 733 (616-1434) | 0.43 |

Data represent GR expression adjusted for GAPDH (mean ± SE).
Cortisol is expressed as median (IQR). P values represent Mann-Whitney U tests.
N= 38 vs 19 for liver, 42 vs 22 for muscle, 47 vs 16 for serum, in patients with conventional or intensive insulin therapy respectively
IQR, Interquartile range

### *Severity of illness and GR expression*

We used the APACHE II score, the TISS-28 score, and serum IGFBP1 levels as different indicators of severity of illness. Higher scores indicate more severe illness and a higher number of therapeutic interventions, respectively. Serum IGFBP1 levels have been shown to have a predictive value for mortality similar to that of the APACHE II score in prolonged critical illness (23). It should be noted that although higher APACHE II and TISS-28 scores and higher IGFBP1 levels indicate a worse prognosis of the patient, and thus may represent a more severe type of illness, all these patients died in the ICU. GR expression was highest, although not signifi-

cantly different, in the livers of patients who died of severe brain damage compared to the other patients (data not shown).

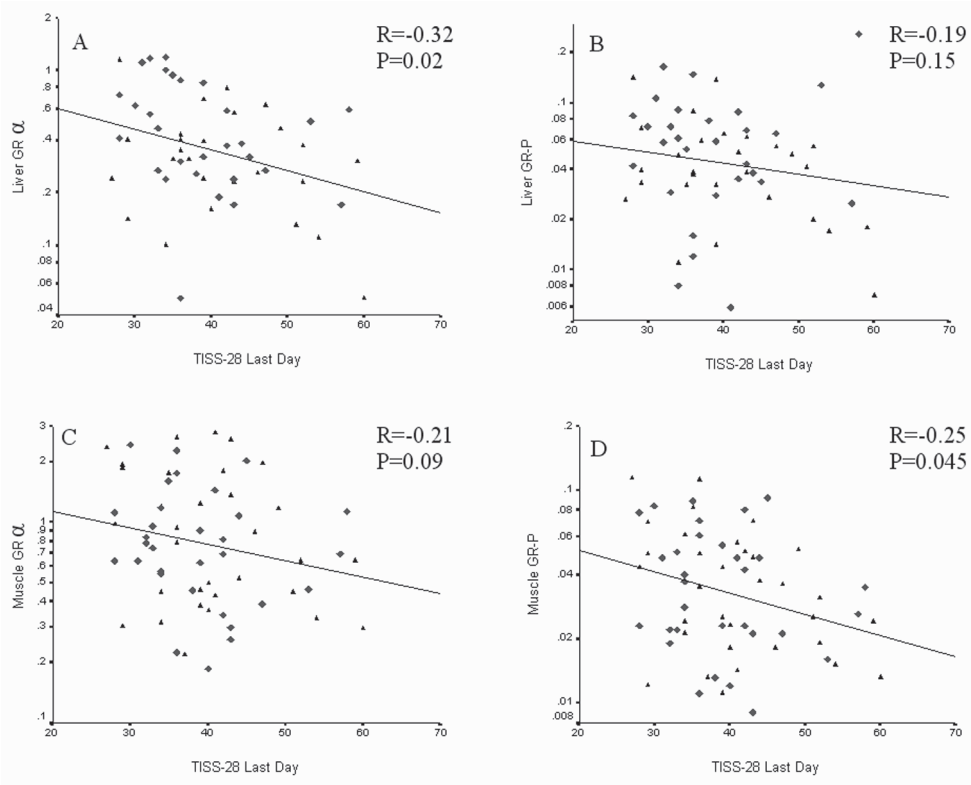


**Figure 2.** Correlation analysis of liver GRα (A), liver GR-P (B), skeletal muscle GRα (C) and skeletal muscle GR-P (D) with the TISS-28 score on the last day. A higher score indicates a higher number of therapeutic interventions. Patients who did not receive glucocorticoid treatment are represented by ●, whereas patients who received glucocorticoid therapy are represented by ▲. R represents Spearman's correlation coefficient.

Liver GRα, but not GR-P, was negatively correlated with the TISS-28 score on the day the patient died (TISS-LD) (Fig. 2), and showed a negative tendency with the APACHE II score on the last day (APACHE-LD). APACHE-LD was not known of 16 patients, resulting in a decreased statistical power. TISS-LD was negatively correlated with skeletal muscle GR-P and GRβ mRNA expression ($P<0.05$ for both), and showed a negative trend with GRα ($P=0.09$) (Fig. 2). APACHE-LD was not correlated with muscle GR expression (Table 4). The GR α/P ratio in both liver and skeletal muscle was unaffected by severity of illness, although the ratio showed a negative trend with the APACHE II score on the last day (Table 4).

**Table 4.** Correlation analysis of GR expression and indicators of severity of illness

| | | APACHE II | TISS-28 | IGFBP-1 |
|---|---|---|---|---|
| Liver GRα | R | -0.27[a] | -0.32 | -0.59 |
| | P | 0.09 | 0.02 | <0.001 |
| Liver GR-P | R | 0.08[a] | -0.19 | -0.31 |
| | P | 0.96 | 0.15 | 0.02 |
| Liver GRα/P | R | -0.30[a] | -0.13 | -0.30 |
| | P | 0.06 | 0.32 | 0.03 |
| Muscle GRα | R | -0.15[b] | -0.21 | -0.14 |
| | P | 0.32 | 0.09 | 0.29 |
| Muscle GR-P | R | 0.07[b] | -0.25 | -0.15 |
| | P | 0.62 | 0.045 | 0.27 |
| Muscle GRβ | R | -0.19[b] | -0.28 | -0.11 |
| | P | 0.25 | 0.045 | 0.48 |
| Muscle GRα/P | R | -0.25[b] | 0.06 | -0.07 |
| | P | 0.09 | 0.65 | 0.58 |

R represents Spearman's Correlation coefficient,
N=57 for liver, N=64 for skeletal muscle. [a] N=41, [b] N=48

Serum IGFBP1 levels on the last day were negatively correlated with liver GRα, liver GR-P, and the liver GRα/P ratio (Table 4), but not with GR expression in skeletal muscle.

### *Cortisol levels and GR expression*

Of only 42 patients both peri-mortem cortisol and liver GR mRNA expression were known, and of those patients 21 were treated with glucocorticoids at some point while they were on the ICU (12 were treated with methylprednisolone, 8 with hydrocortisone, and one patient received both treatments). Of 49 patients both peri-mortem cortisol and skeletal muscle GR mRNA expression were known, and of those patients 26 were treated with glucocorticoids at some point while they were on the ICU (14 were treated with methylprednisolone, 11 with hydrocortisone, and one patient received both treatments).

Serum peri-mortem cortisol levels showed a negative, but not significant, trend with liver GRα (R= -0.28, P=0.08), liver GR-P (R= -0.19, P=0.29) and with skeletal muscle GRα mRNA expression (R= -0.23, P=0.12). After the exclusion of one outlier

(Fig. 3), the negative relation between liver GRα expression and cortisol levels was significant (R= -0.34, P=0.03). Skeletal muscle GR-P was also negatively correlated with peri-mortem cortisol (R= -0.32, P=0.02) (Fig. 3), but failed to reach significance if it was expressed relative to the HPRT house keeping gene instead of GAPDH.

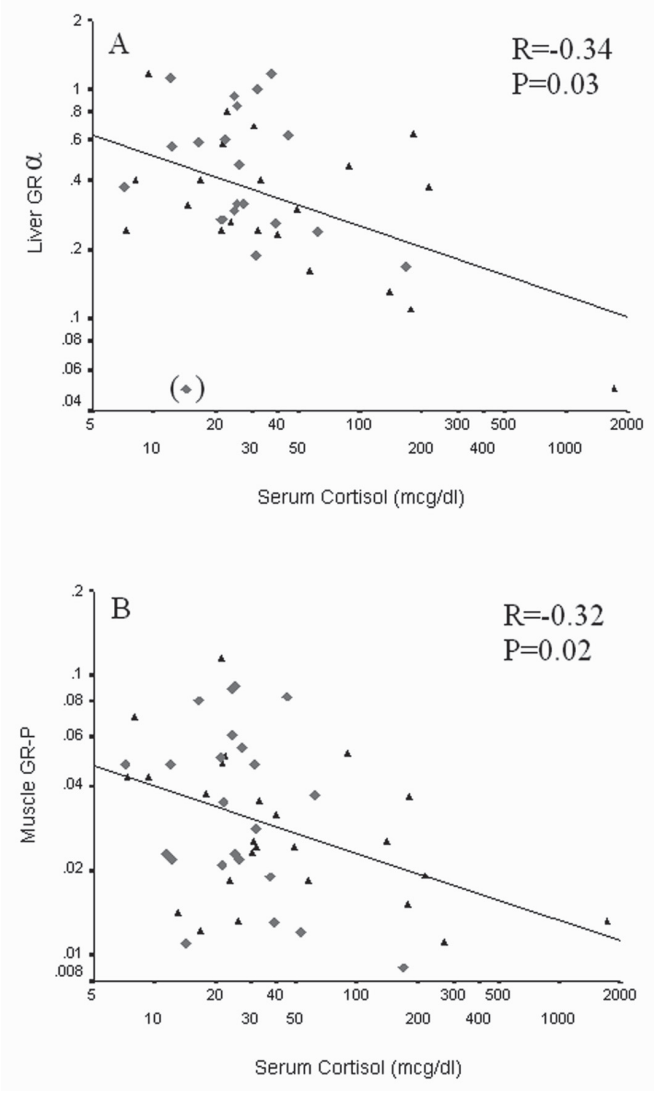


**Figure 3.** Correlation analysis of liver GRα (A), and of skeletal muscle GR-P expression (B) with peri-mortem serum cortisol levels. Patients who did not receive glucocorticoid treatment are represented by ●, whereas patients who received glucocorticoid therapy are represented by ▲. R represents Spearman's correlation coefficient. Correlation coefficient and P-value shown in figure (A) are calculated after the exclusion of the one outlier, as indicated by the brackets.

Serum cortisol levels at 6.00 am on the last day, and also the calculated free cortisol levels, showed similar negative relations as the peri-mortem cortisol levels, but failed to reach significance (R=-0.25, P=0.12 for liver GRα, R=-0.26, P=0.11 for GR-P, R=-0.18, P=0.21 for muscle GRα, and R=-0.26, P=0.08 for GR-P), suggesting that peri-mortem cortisol levels in these patients correspond better to post-mortem GR mRNA levels than morning cortisol (and morning free cortisol) levels on the last day.

### *GC treatment and GR expression*

Liver GRα and GR-P mRNA expression tended to be lower in patients who were treated with glucocorticoids at any moment while they were on the ICU (N=28) as compared with patients who did not receive glucocorticoids (P=0.05 for GRα and P=0.09 for GR-P). This difference was accentuated in patients who were treated with glucocorticoids until the day they died (N=16), since these patients had significantly lower levels of GR mRNA expression as compared to patients who did not receive this treatment on the last day (P=0.006 for GRα, and P=0.001 for GR-P) (Fig. 4A, C). Skeletal muscle GR mRNA expression showed no difference between patients treated and patients not treated with glucocorticoids (Fig. 4B, D), nor was there any effect on the α/P ratio in liver or skeletal muscle.

### *Immunoblotting*

Immunoblotting of the GR protein with the N-terminal antibody PA1-511A in 12 liver homogenates showed two bands, also present in COS cells transfected with the GRα plasmid, and probably reflecting the A- and the B- translation variants of the GRα protein (26). No additional bands, possibly representing the GRβ or GR-P splice variants, were detected. Immunoblotting of the GRα protein in liver samples corresponded with the data on GRα mRNA expression in the samples, as is illustrated in Fig. 5. Immunoblotting of the GR protein in skeletal muscle did not result in any specific bands.

## Discussion

This is the first study examining the expression of splice variants of the glucocorticoid receptor during critical illness. Analysis of tissue samples obtained from ICU patients within minutes after death demonstrates the presence of GRα and GR-P mRNA in liver and of GRα, GRβ, and GR-P mRNA in skeletal muscle. The GRα/GR-P ratio was 2.5 times lower in liver than in skeletal muscle. GRα mRNA expres-

sion per µg RNA in skeletal muscle was approximately 10 times higher than in liver, which corroborates previous findings in healthy individuals, where GRα mRNA expression was 3 times higher in skeletal muscle than in liver (27).

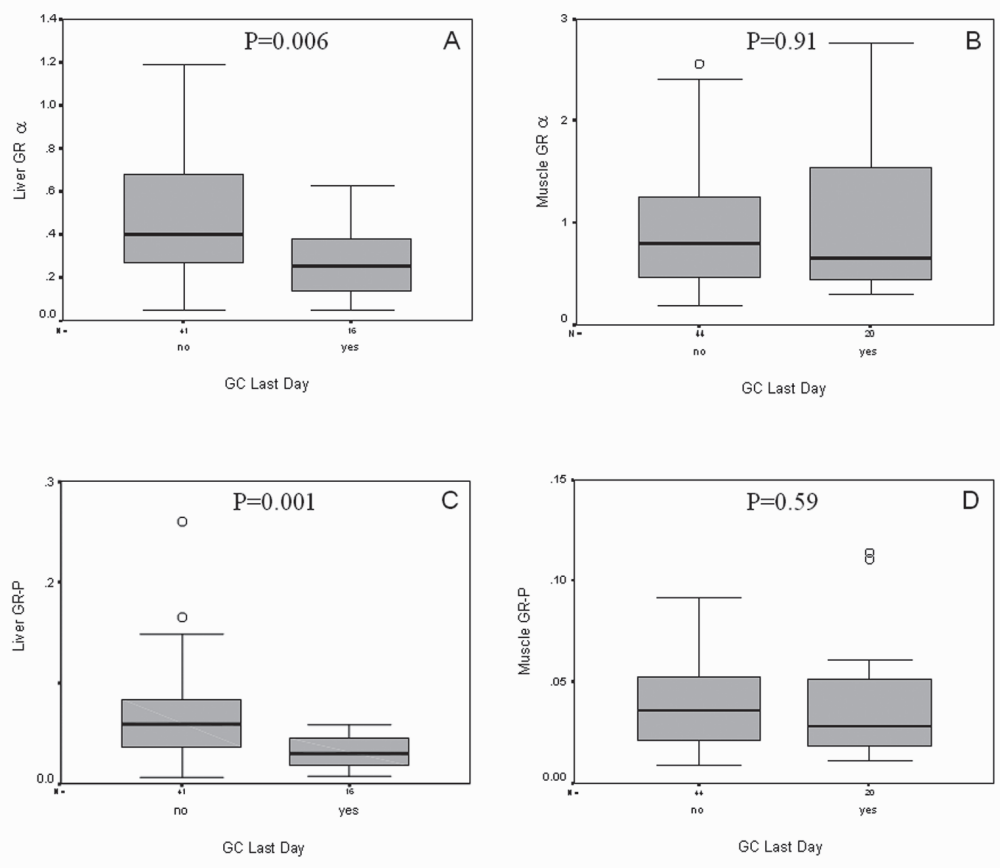


**Figure 4.** Liver GRα and GR-P mRNA expression in patients who were treated with glucocorticoids on the day they died was significantly lower than in patients who did not receive this treatment on the last day (Fig. 4A, C). No difference was observed in skeletal muscle GR expression (Fig. 4B, D). Box plots represent 10th-25th-50th-75th-90th percentiles, and P values were obtained with Mann-Whitney U test. Open circles represent outliers.

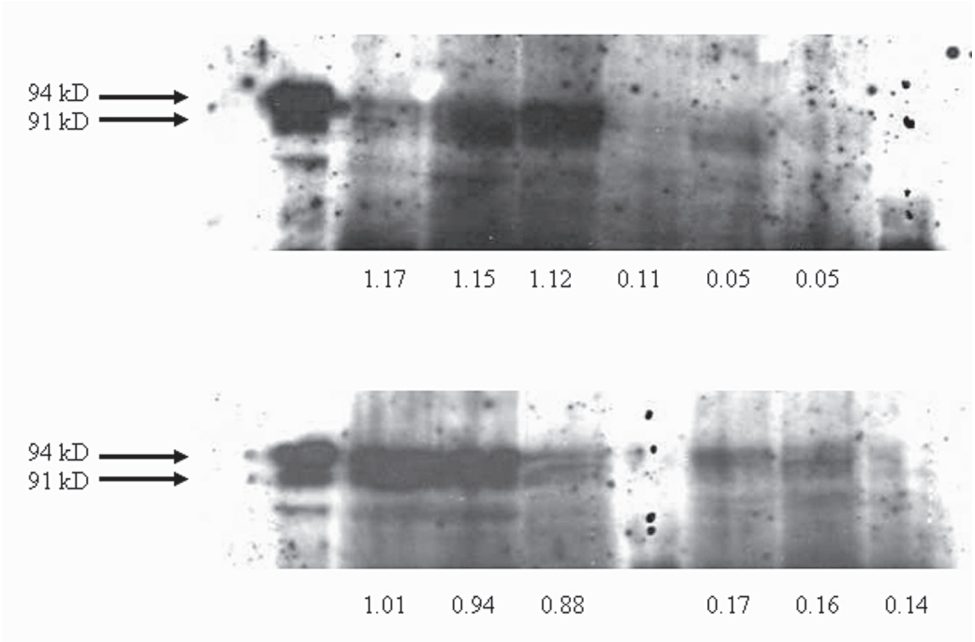


**Figure 5.** Immunoblotting of the GRα protein in a selection of liver homogenates, using the N-terminal antibody PA1-511A. COS cell homogenates transfected with the previously described pRShGRα plasmid were used as a positive control (left lane). GRα mRNA expression, as was measured by real time RT-PCR in that liver sample, is shown on the bottom of each lane. Each blot shows three samples with a high GRα mRNA expression on the left, and three samples with a low GRα mRNA expression on the right. These data illustrate that GRα mRNA and protein levels correlate, although there is a large variation among the different samples. The upper band represents the translation variant A of GRα, whereas the lower band represents the translation variant B.

GRβ mRNA expression was undetectable in all but 5 livers, and in skeletal muscle GRβ was ~ 10,000 times lower than GRα. This is in agreement with previous studies, in which very little GRβ mRNA was found compared to GRα in different tissues and tumor cells (13, 16, 27-31). However, the GR β/α ratio is generally higher at the protein level than at the mRNA level (27, 32-34), which might be due to the longer half-life of the GRβ protein (35, 36). Despite the low levels of GRβ mRNA, the GRβ protein seems to be widely expressed in the normal situation (34, 37, 38). Nevertheless, in this study we were not able to detect the GRβ protein in a subset of patients.

Activation of the HPA-axis is an essential adaptive mechanism to severe physical stress. The high cortisol levels have a major effect on glucose metabolism, by stimulating hepatic gluconeogenesis and by inducing peripheral insulin resis-

tance. In this respect, liver and skeletal muscle are target tissues. GR mRNA expression was not different between patients treated with intensive or conventional insulin therapy, nor was there any effect on the ratio of the different variants. This cannot be interpreted as indicating that the beneficial effects of intensive insulin therapy on both morbidity and mortality in intensive care patients are not related to changes in GR expression, however. The current study only involved post-mortem tissue samples, making a selection bias (sicker patients in the studied group receiving intensive insulin therapy) not unlikely. Hence, an effect of insulin therapy on GR expression or cortisol levels in survivors of the large insulin-in-ICU study cannot be excluded.

A strong positive linear relation was observed between the expression of the different GR variants, both in liver and in skeletal muscle. This indicates that in critically ill patients GRα, GRβ, and GR-P vary in parallel, suggesting that there is no specific differential regulation of alternative splicing of the GR in critical illness. This is supported by the observation that different indicators of severity of illness show a negative relation with GR expression, but not with the α/P and/or α/β ratio. Higher APACHE II and TISS-28 scores, which indicate more severe illness and a higher number of therapeutic interventions respectively (20-22), were associated with or showed a tendency towards a lower expression of liver GR and muscle GR mRNA expression. Circulating IGFBP-1, which has been shown to be a negative predictor of clinical outcome in critical illness (23), showed a significant negative relation with liver, but not muscle GR expression.

In critically ill patients, also serum cortisol levels correlate with the severity of illness. They are an independent predictive factor for clinical outcome, and they reflect the intensity of the activation of the HPA-axis (4, 39, 40). In this study, serum peri-mortem cortisol levels showed a negative association with liver GRα and with skeletal muscle GR-P mRNA expression, whereas morning (free) cortisol levels showed a negative trend. This negative relation of mRNA expression of the GR variants, but not of their ratios, with cortisol and with different indicators of severity of illness, suggests a down-regulating effect of HPA-activation on GR expression. This is supported by an approximately 3 times higher expression of GR, with a similar α/P ratio in livers of 4 relatively healthy subjects (data not shown), and also by the observation of a higher, although not significant, expression of GR in the livers of patients who died of severe brain damage compared to the other patients. Down-regulation of tissue GR expression may result from chronic exposure to increased cortisol levels and may counteract the consequential catabolic effects in patients with prolonged critical illness.

The value of corticosteroid therapy in patients with sepsis or septic shock remains controversial (41-44), although recent studies have reported substantial benefits of stress dose corticosteroid replacement during septic shock (19, 45-47). In this study, both liver GRα and liver GR-P mRNA expression, but not skeletal muscle GR expression, were substantially lower in patients who received exogenous glucocorticoids on the day they died than in patients who did not receive this treatment. These data suggest that glucocorticoid treatment down-regulates the expression of the glucocorticoid receptor, and thus glucocorticoid sensitivity, in the liver. This is in agreement with in vitro studies, which have shown that glucocorticoids can induce a homologous down-regulation of GR expression through inhibition of GR transcription (48) and reduction of GR mRNA stability (49). Interestingly, no such effect was observed in skeletal muscle, which suggests that the down regulating effect of glucocorticoids may be tissue specific and that the risk of steroid induced insulin resistance, catabolism and dysfunction in muscle persists during pharmacologically high levels of cortisol.

Our data regarding hepatic GRα mRNA expression were substantiated by a correlation with liver GRα protein expression in a subset of samples. This suggests that the changes in liver GRα expression in severely ill patients are at least in part exerted at the pretranslational level. Unfortunately, due to a limited sensitivity, we were not able to detect the GRβ or GR-P splice variants in liver and/or GR protein expression in skeletal muscle samples of these patients.

In conclusion, analysis of tissue samples of patients who died in the ICU demonstrated the presence of GRα and GR-P mRNA in liver and of GRα, GRβ and GR-P mRNA in skeletal muscle. The mRNA levels of the different variants, both in liver and in skeletal muscle, were positively correlated with each other. The negative relation of mRNA levels of the receptor variants with circulating cortisol and severity of illness suggests a down-regulating effect of HPA-activation on GR expression. In contrast to liver GR expression, muscle GR expression was not lower in patients receiving exogenous glucocorticoids. This might imply that muscle tissue is less sensitive to the downregulating effects of glucocorticoids in critical illness and may predispose patients to the negative side effects of glucocorticoid treatment.

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# *General Discussion*

The studies in this thesis consist of two major parts. In the first part (**Part A**), we have analyzed the genetic variation in thyroid hormone pathway genes. We have shown that polymorphisms in thyroid hormone pathway genes are associated with serum TSH and iodothyronine levels in healthy subjects of different age. Furthermore, we have shown that these polymorphisms are not only related to serum levels of thyroid hormone, but also to several thyroid hormone-related endpoints, such as insulin resistance and atrial fibrillation. The new insights that can be obtained from these studies about the physiological role of the analyzed genes, are discussed in Part A of the general discussion. Also the clinical consequences that these studies may have in the (near) future, are discussed in this section.

In the second part of this thesis (**Part B**), we have analyzed the regulation of thyroid hormone bioactivity during critical illness. Pronounced alterations in serum thyroid hormone (sulfate) levels occur during critical illness, and we have shown that these alterations are (partially) due to a decreased activation of thyroid hormone by D1 and by an increased inactivation by D3. We have also shown that higher levels of rT3 and a lower T3/rT3 ratio are associated with a worse prognosis, and that these changes also occur at the tissue level. The importance and possible consequences of these data are discussed in Part B of the general discussion. In addition, we analyzed the regulation of cortisol bioactivity in the addendum of this thesis.

A logical bridge between Part A and Part B of this thesis would be to study the role of genetic variation during critical illness. A major advantage of such a study would be that not only cDNA samples and serum hormone levels of the critically ill patients are available, but we have also measured enzyme activities and deiodinase mRNA levels in these patients. In this way, we will not only be able to analyze the association of deiodinase polymorphisms with serum levels, but also directly with enzyme activities. Furthermore, we will be able to investigate if possible changes in enzyme activities are related to changes in mRNA levels.

Yet, there are also reasons to expect little if any effect of the genetic variants in critical illness. The above studied polymorphisms all seem to result in very subtle changes in enzyme activities. Although these subtle changes may be very important in the normal situation, since they result in altered thyroid hormone activity throughout life, this may very well be different in an intensive care setting. In the critically ill patients we have studied, there is an enormous variation between the patients. This is a logical consequence of analyzing samples of different patients, with varying kinds and severity of illness, who all received various forms of medical treatment in an intensive care setting. Thus, the subtle effects of genetic variation could be masked by the large variety in diseases and treatments of the population. Further research will be necessary to evaluate the role of polymorphisms in thyroid hormone pathway genes during critical illness.

# *General Discussion*

## *Part A*

### Genetic variation in thyroid hormone pathway genes

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

*Submitted*

## Introduction

Thyroid hormone is essential for growth and differentiation, for the regulation of energy metabolism, and for the physiological function of virtually all human tissues (1, 2). The production of thyroid hormone is regulated by the classic hypothalamus-pituitary-thyroid axis, whereas the biological activity of thyroid hormone, i.e. the availability of T3 for the nuclear thyroid hormone receptors, is mainly regulated at the tissue level by the three different iodothyronine deiodinases and thyroid hormone transporters (3-5).

In healthy subjects, serum thyroid parameters show a substantial inter-individual variability, whereas the intra-individual variability is within a narrow range (6, 7). This suggests an important influence of genetic variation on the regulation of thyroid hormone bioactivity, resulting in a thyroid function set-point that is different for each individual. This notion is supported by a classical twin study that was recently published (8). In this study, heritability accounted for ~ 65% of the variation in serum TSH, FT4 and FT3 levels. In a Mexican-American population, total heritability in serum thyroid parameters ranged from 26–64% of the total inter-individual variation observed, whereas the effects of environmental and lifestyle covariates accounted for only 2–18% of the total phenotypic variation (9).

Findings in patients with subclinical hyper- and hypothyroidism illustrate that already minor alterations in thyroid hormone levels (and in thyroid hormone bioactivity) can have important consequences for a variety of thyroid hormone-related clinical endpoints, such as atherosclerosis, bone mineral density, obesity, and heart rate (10-12). In the last few years, several studies described polymorphisms in thyroid hormone pathway genes that (may) result in an altered thyroid hormone bioactivity. Some of these polymorphisms are associated with serum TSH and/or thyroid hormone levels in healthy subjects, and/or with thyroid hormone-related clinical endpoints. Since DNA variations are stable throughout life, such genetic effects are likely to have an influence during the lifetime of subjects.

In this review, we discuss the genetic variation in thyroid hormone pathway genes, focussing on the polymorphism studies that have emerged in the last few years. For the sake of brevity, we have only focussed on single nucleotide polymorphisms (SNPs) in the TSH receptor, iodothyonine deiodinases, and thyroid hormone transporters and receptors. We will discuss the possible consequences of these studies for the individual patient, and also the new insights about thyroid hormone action that can be obtained from these data.

### *Polymorphisms in the TSH receptor*

Many somatic gain-of-function mutations in the TSH receptor (TSHR) have been described, resulting in a phenotype of toxic adenoma or toxic multinodulair goiter (see refs (13, 14) for reviews). Also germline gain-of-function TSHR mutations have been described, resulting in congenital hyperthyroidism. Conversely, germline loss-of-function TSHR mutations are associated with TSH resistance and congenital hypothyroidism (see refs (15-17) for illustrative examples). Only three germline TSHR polymorphisms, resulting in amino acid substitutions, have been identified, however (18-20). Two of them are located in the extracellular domain of the receptor (Asp36His and Pro52Thr) (18, 19), and one is located in the intracellular domain (Asp727Glu) (20) (Fig. 1). In a population of healthy blood donors, the TSHR-Glu727 polymorphism was associated with lower levels of plasma TSH, but had no effect on FT4 (21). We have recently confirmed this observation in an unrelated study population (unpublished data), but not in a population of elderly men of > 70 years old (22). The lower TSH but similar FT4 levels *in vivo* agree with *in vitro* findings by Gabriel et al., who showed an increased cAMP response of the TSHR-Glu727 variant to TSH (20), since less TSH would be required to achieve a normal thyroid hormone production. It should be noted, however, that other studies have so far not been able to reproduce these *in vitro* data, which could be due to different cell types that were studied (23, 24). Conflicting data are also available regarding the response of the TSHR-Pro52Thr variant to TSH stimulation (25-27), which could reflect the subtle effects of these polymorphisms. The TSHR-Pro52Thr and -Asp36His polymorphisms were not associated with changes in serum TSH or iodothyronine levels in healthy blood donors. However, this could be due to the low allele frequency of these SNPs (6% and 0.6%, respectively) resulting in a lack of power. In addition, several intronic microsatellite markers and intronic SNPs have been described in TSHR (28-32).

The *TSHR* gene is located on chromosome 14q31, an area in which also a Graves' disease susceptibility locus (GD-1) has been mapped (33). The GD-1 locus is specifically linked to Graves' disease, but not Hashimoto's thyroiditis or autoimmune thyroid disease in general. Several case-control studies have been carried out analyzing the possible association of one or more of the previously mentioned TSHR polymorphisms and autoimmune thyroid disease. An overview of the 14 studies that analyzed the possible association of TSHR polymorphisms with Graves' disease up to 2002 is presented by Ban et al. (34). Six studies focused on the TSHR-Pro52Thr polymorphism, four on TSHR-Asp727Glu, four on microsatellite markers in introns 2 and/or 7, and one on TSHR-Asp36His (20, 28, 31, 34-44). All studies

analyzing the TSHR-Pro52Thr or Asp36His variant were negative (35-40), except the oldest one in which an association of the TSHR-Pro52Thr variant with Graves' disease was described in US Caucasian females (N=100 females with autoimmune thyroid disease vs. 69 controls) (35). These same authors have later described two subjects who were homozygous for the Thr52 allele and had a normal thyroid function, on the basis of which they suggested that the variant receptor is able to respond normally to TSH (25). Obviously, more subtle effects of this polymorphism cannot be excluded from the last study. In a multiethnic (Chinese, Malays, and Indians) cohort of patients with Graves' disease, TSHR-Asp36His was absent, and TSHR-Pro52Thr and -Asp727Glu were not associated with Graves' disease (45). Unfortunately, no data are currently available whether the two variants in the extracellular domain of the receptor show an altered binding of thyroid- stimulating antibodies. Nor are any data available of an altered binding or altered cAMP response of the variant TSH receptors to a different TSHR ligand that has recently been identified, named thyrostimulin (46, 47).

Three case-control studies in Caucasians showed no association between the TSHR-Asp727Glu polymorphism and Graves' disease (20, 34, 42). Meta-analysis of these three studies, however (34), as well as a study in Russian patients (N=78 vs. 93 controls) showed a weak association of the variant receptor with Graves' disease (41, 48). These patients had a significantly higher frequency of the TSHR-Glu727 allele than healthy subjects (41, 48). A recent transmission disequilibrium test (TDT) study in Russian families showed that the D2-Thr92Ala (see below) and TSHR-Asp-727Glu polymorphisms are in weak linkage disequilibrium (49), and that the D2-Ala92/TSHR-Glu727 haplotype allele was preferentially transmitted from parents to affected siblings with Graves' disease (49). However, TDT is not the best design to analyze linkage disequilibrium. A recent study in Japanese patients with autoimmune thyroid disease showed that several SNPs in intron 7 of the TSHR gene are significantly associated with Graves' disease (30). Another polymorphism in intron 4 of the TSHR gene was associated with Graves' disease in a multiethnic population of patients from Singapore (45). These data together suggest that genetic variation in *TSHR* is, albeit weakly, associated with the development of Graves' disease, but it is yet unclear whether the associated polymorphisms are functional or whether they are linked to functional variants elsewhere in the gene that still have to be discovered. Detailed linkage disequilibrium analysis and haplotype tagging approaches (as well as the HapMap project) should be able to resolve this issue.

TSHR is not only expressed in the thyroid, but also, among other tissues, in adipose tissue (50, 51), brain (52, 53), orbital tissue (51, 54-56), lymphocytes (57),

and bone (58). More and more evidence becomes available of direct effects of TSH via the TSHR on these tissues. TSH is able to induce proliferation and inhibit differentiation in cultured rat pre-adipocytes (59), and TSHR knock-out mice have a severe phenotype of osteoporosis, independent of their thyroid hormone levels (58). Genetic variation in the TSH receptor may thus not only be important for the development of autoimmune thyroid disease (more specifically Graves' disease), but also for more common clinical endpoints such as insulin resistance and osteoporosis (22, 60). However, to our knowledge no case-reports about additional phenotypes in patients with germline gain- or loss-of-function TSHR mutations are available.

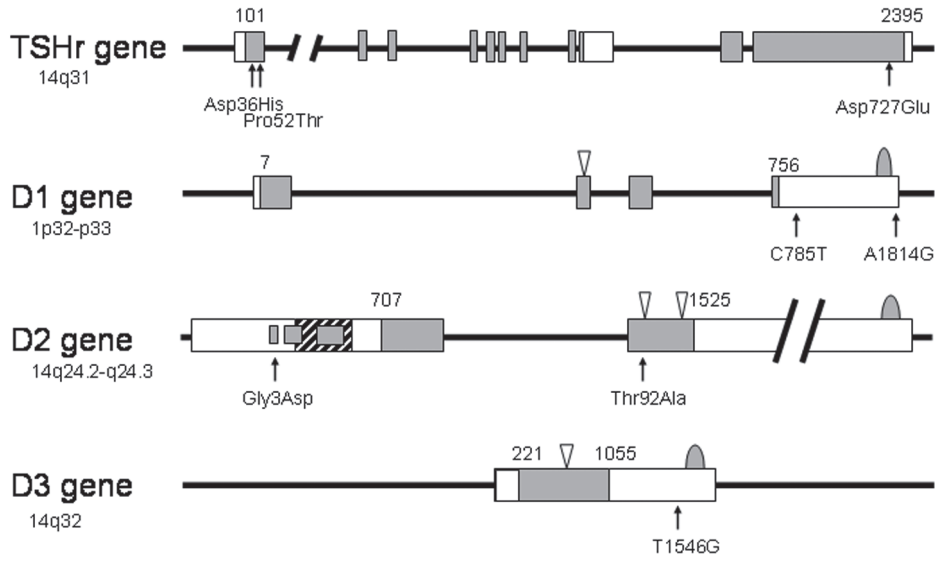


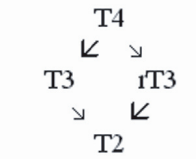
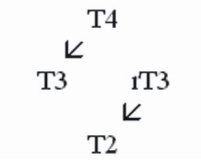
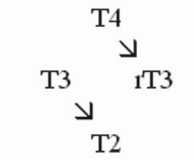

**Figure 1.** Exonic polymorphisms in the TSH receptor and the deiodinases that are described in this study. The coding sequence is represented by ▭, the 5'- and 3'-UTR by ▭, whereas ▨ represents an alternatively spliced exon. A UGA codon, coding for SeC, is depicted by ▽. Finally, SECIS elements are indicated by ◠.

## *Polymorphisms in the iodothyronine deiodinases*

No patients with inactivating mutations in any of the iodothyronine deiodinases have yet been described. Whether this means that these mutations are not compatible with life, that they have little or no consequences, or that they result in unexpected, yet unknown phenotypes is unclear. Based on the phenotypes of mice with targeted deletions of D1, D2 or D3, the most severe effects would be expected of mutations in D3 (61-63). Nevertheless, mice have drawbacks as models of humans. In the last few years, several polymorphisms in the deiodinases have been

described (21, 64, 65) (Fig.1). Based on the physiological role of the three different deiodinases (3) (Table 1), one can speculate about the possible consequences of the polymorphisms in these enzymes. D1 is present in liver, kidney, and thyroid, and plays a key-role in the production of the active hormone T3 from T4 and for the clearance of the metabolite rT3 (3, 4). D2 is present in brain, pituitary, brown adipose tissue, thyroid, skeletal muscle, aortic smooth muscle cells, osteoblasts, and D2 mRNA has also been detected in the human heart (3, 66-69). In tissues such as the brain, D2 is important for local production of T3, whereas D2 in skeletal muscle may also contribute to plasma T3 production. D3 is present in brain, skin, placenta, pregnant uterus, various fetal tissues, and is induced in liver and muscle during critical illness (3, 4). D3 is the major T3 and T4-inactivating enzyme and contributes to thyroid hormone homeostasis by protecting tissues from excess thyroid hormone. The serum T3/rT3 ratio is relatively independent of thyroidal T4 production and of variations in serum binding proteins. The T3/rT3 ratio is considered to be a good reflection of the peripheral metabolism of thyroid hormone, being positively influenced by D1 and D2 and negatively by D3. This ratio is also relatively independent of thyroidal T4 production and of variations in serum binding proteins.

**Table 1.** Physiological role in thyroid hormone metabolism, tissue distribution, and substrate preference of the three human iodothyronine selenodeiodinases (D1-D3).

| | D1 | D2 | D3 |
|---|---|---|---|
| |   |   |   |
| Function | plasma T3 production, rT3 clearance | local and plasma T3 production | T3 and T4 clearance, rT3 production |
| Tissue distribution | Liver, kidney, thyroid | Brain, pituitary, BAT, thyroid, skeletal muscle, heart, aortic smooth muscle, osteoblasts | Brain, skin, placenta, fetal tissues, critically ill liver and skeletal muscle |
| Substrate preference | rT3>>T4=T3 | T4>rT3 | T3>T4 |

## *D1*

Recently, two polymorphisms in D1, affecting the serum T3/rT3 ratio in healthy subjects, were identified (21) (Fig.1). D1a-C785T was associated with higher levels of rT3 and with a lower T3/rT3 ratio. Based on the function of D1 (Table 1), it was speculated that D1a-C>T results in a decreased activity of D1. D1b-A1814G was associated with a higher T3/rT3 ratio, suggesting that D1b-A>G may result in an increased activity (21). Because both SNPs are located in the 3'-UTR of the mRNA, a change in the stability of the mRNA is an attractive explanation for their effect. Alternative explanations may be an altered folding of the mRNA, in particular of the selenocysteine insertion sequence (SECIS), which is necessary for the incorporation of a selenocysteine residue in the catalytic center of the protein (3), or that these SNPs are in linkage disequilibrium with other polymorphisms in the coding sequence or in regulatory areas of the gene. Functional testing and haplotype analysis will be necessary to resolve this issue. Although D1a-C>T was not associated with serum rT3 levels in a population of 350 men (age > 70 years), its association with lower levels of T3 in this elderly population supported the theory of a lower activity of D1 in carriers of this polymorphism (70). The different associations found in the healthy blood donors and the elderly men might be explained by the difference in age, with means of 46 vs. 77 years. In young subjects, a decreased T3 production by D1 may be masked by the production of serum T3 by skeletal muscle D2. Throughout adult life, skeletal muscle size and strength gradually decline, resulting in a decrease in D2-expressing skeletal muscle. Furthermore, rT3 levels increase with age, and degradation of the D2 protein is accelerated when it is exposed to its own substrates T4 and rT3 (71). Although it has been shown that D1 activity also decreases during aging (72, 73), the relative contribution of D2 to serum T3 production may be less important in elderly than in young subjects. This would mean that D1 has a relatively greater contribution to serum T3 production at advanced ages (70) (Fig. 2). In line with this hypothesis is the recent publication by our group of a polymorphism in a short open reading frame (ORFa) in the 5'-UTR of D2, which has been shown to be an important regulatory element (65, 74) (Fig. 2). This polymorphism (D2-ORFa-Gly3Asp) is associated with the serum T3/T4 ratio in younger, but not in elderly subjects (65). Also supporting this hypothesis is the association of D1a-C/T with both serum T3 and rT3 levels in an unrelated third population, with an average age of 69 years old (unpublished observations).

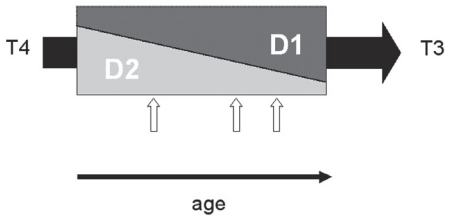



**Figure 2.** Illustration of the proposed model in which the relative contribution of D2 to serum T3 production decreases with an increase in age, based on the different associations of D1 and D2 polymorphisms with serum iodothyronines in one younger (left arrow) and two elderly populations (right two arrows).

Haplotype analysis showed that the D1a-C>T and D1b-A>G polymorphisms appear on different haplotype alleles (21, 70). The haplotype allele containing D1a-T is not only associated with changes in serum iodothyronine levels but also with increased levels of free insulin-like growth factor-I (IGF-I) in two unrelated populations (70). This was substantiated by the association of this haplotype allele with several IGF-I related endpoints, such as increased muscle strength and muscle mass (70). Since IGF-I has a stimulatory effect on D1 expression (75), these higher levels of free IGF-I may be seen as an adaptation to normalize D1 activity in carriers of the D1a-T haplotype allele. On the other hand, thyroid hormone stimulates the expression of IGF-binding protein-I (IGFBP-I) in human hepatoma cells (76). A lower activation of thyroid hormone by liver D1 could result in lower levels of IGFBP-1, and thus a higher level of free IGF-I (70), since IGFBP-1 is mainly produced in the liver (77).

### *D2*

D2 is important in the production of local T3, but D2 in skeletal muscle also contributes to serum T3 production. The above-mentioned association of D2-ORFa-Gly3Asp with the serum T3/T4 ratio also points towards an important role of D2 in serum T3 production, which may decrease with an increase in age (65, 70). The first polymorphism described in any of the deiodinases was D2-Thr92Ala (64) (Fig. 1). Although this polymorphism does not seem to be associated with serum iodothyronine levels, it has been associated with insulin resistance in three different populations (22, 64, 78). The mechanism behind this association is yet unclear, but it may involve expression of D2 in skeletal muscle and/or in (brown) fat in humans (3, 67). T3 stimulates the transcription of the muscle/fat-specific insulin-sensitive glucose transporter GLUT4 (79-82). Furthermore, thyroid hormone augments cat-

echolamine-stimulated lipolysis (83), and a particular inactivating thyroid hormone receptor alpha mutation results in a phenotype of insulin resistance in mice (84). A decreased D2 activity in insulin-sensitive tissues such as adipose tissue and skeletal muscle, resulting in a decreased availability of local T3, may thus explain the association of D2-Thr92Ala with relative insulin resistance (22, 64, 78). Alternatively, hypothalamic D2 regulating T3 content of brain stem neurons projecting to white adipose tissue may be involved (85). Although D2 activity is not different between different cells that are transfected with the D2-92Ala variant or the wild-type variant (21, 78), a lower activity of D2 has been reported in muscle and thyroid homogenates of carriers of the D2-Ala92 allele (78). This might suggest that the possible consequences of the D2-Thr92Ala polymorphism are due to linkage with another polymorphism.

Haplotype analysis has shown that the D2-Thr92Ala polymorphism and the previously mentioned D2-ORFa-Gly3Asp polymorphism appear on different haplotype alleles (65). So far, there is no evidence for any relation of the D2-ORFa-Gly3Asp polymorphism with insulin resistance. An alternative candidate to explain the above mentioned association would be the previously mentioned TSHR-Asp-727Glu polymorphism, but this is unlikely for genetic reasons. The D2 gene and TSHR gene are both located on chromosome 14, separated by a distance of more than 700,000 basepairs with their 5'-UTRs facing inward. In addition, D2-Thr92Ala and TSHR-Asp727Glu have been shown to be in weak linkage disequilibrium (49). Yet, interestingly, TSHR-Asp727Glu is also associated with insulin resistance, indicating possible independent effects of these two genes (22). Indeed, regression analyses of models incorporating both genes and looking at relative insulin resistance suggested independent and additive effects of the two polymorphisms (22). The association of TSHR-Asp727Glu with insulin resistance may involve expression of the TSH receptor in adipocytes (22, 51), since TSH is able to induce proliferation and inhibit differentiation of cultured rat preadipocytes (59). A more active TSH receptor may thus result in an increased adipogenesis, leading to an increased fat mass and making subjects more vulnerable to the development of insulin resistance (22).

Based on the expression pattern of D2, and since D2 is crucial in the regulation of local T3 concentrations, one can speculate about other possible consequences of these and other D2 polymorphisms. Guo et al. studied the relation of the *DIO2* gene with mental retardation in iodine-deficient areas of China in a case-control study (N=96 vs. 331 controls) (86). They found a positive association of two intronic D2 polymorphisms (but not of D2-Thr92Ala or of TSH receptor polymorphisms)

with mental retardation in these areas (86, 87), and concluded that genetic variation in D2 may determine the risk of developing mental retardation in an iodine deficient area, probably by affecting the local amount of T3 available in the brain (86, 87). Appelhof et al. addressed the questions whether genetic variation in D2 is a determinant of well-being and neurocognitive functioning in hypothyroid patients on levothyroxine substitution, and whether D2 polymorphisms were associated with a preference for T4/T3 combination therapy over substitution with T4 alone (88). No differences in well-being, neurocognitive functioning or appreciation of T4/T3 combination therapy were detected in these thyroid hormone-replaced hypothyroid patients (88). The expression of D2 in the human heart (67), and the observation that overexpression of D2 in the mouse heart causes cardiac-specific thyrotoxicosis, including/as well as an increased heart rate (89), might suggest that genetic variation in D2 may be important in the development of tachycardia and/or atrial fibrillation. Indeed, a recent study by our group in over a thousand elderly subjects showed a positive association of D2-ORFa-Asp3 with atrial fibrillation, whereas D2-Ala92 showed a negative relation (90).

### *D3*

Until now, only one polymorphism has been identified in D3 (D3-T1546G), located in the 3'-UTR (21). This polymorphism did not result in altered thyroid hormone levels in healthy individuals. D3 plays an important role in thyroid hormone homeostasis in critical illness and during fetal development, providing protection against thyroid hormone excess (91, 92). Possible effects of this polymorphism on development and under pathophysiological conditions remain therefore to be investigated in future studies. A major obstacle in these studies is that the D3 gene is an imprinted gene, with preferential expression from the paternal allele (61). Therefore, the effects of polymorphisms in the *DIO3* gene on thyroid hormone homeostasis depends on the parental origin of the variant allele.

## *Polymorphisms in thyroid hormone transporters and receptors*

In addition to peripheral metabolism of thyroid hormone by the deiodinases, transmembrane transport of iodothyronines is another important process in the regulation of thyroid hormone bioactivity (5). Recently, human MCT8 was identified as the first known thyroid hormone-specific transporter (93, 94), with a preference for T3 over T4 (unpublished data). MCT8 is expressed, among other tissues, in brain (more specifically in neurons), liver, thyroid, and heart, and inactivating mutations in MCT8 result in a phenotype of severe mental retardation (94). A second interest-

ing thyroid hormone transporter is OATP1C1, which is considered to be a brain-specific T4 transporter (95). In rats, it is localized in brain capillaries, and it may therefore be important for transport of T4 across the blood brain barrier (96, 97). To our knowledge, no studies have yet been published investigating the association of polymorphisms in these transporters with clinical endpoints. Based on the important role of thyroid hormone in brain function and development, and based on the distribution of these two known transporters, it is very tempting to speculate about possible associations with, for example, cognition or dementia.

The biological activity of thyroid hormone is determined by T3-dependent gene expression, which is mediated via the different T3 receptor (TR) isoforms, derived from two genes (98, 99). For other nuclear hormone receptors, such as the glucocorticoid and estrogen receptor, many data are available of functional polymorphisms having important clinical consequences (see refs. (100, 101) for two examples). In contrast, hardly anything is known about (functional) polymorphisms in the TRs. Ganly et al. have identified four polymorphisms in the TRβ by restriction fragment length polymorphism (RFLP) analysis 15 years ago (102-105), but hardly any data are available on the exact location and/or possible consequences of these polymorphisms. One study describes the association of a HindIII RFLP that is associated with Graves' disease, but not with autoimmune thyroid disease in general (106). In addition, a non-synonomous SNP in TRβ was described that was not associated with serum iodothyronine levels in healthy subjects (21). In conclusion, this area of research is not well explored, and it is likely that exciting new insight will be obtained in the upcoming years.

## Concluding remarks and future perspectives:

Here we have discussed several polymorphisms in thyroid hormone pathway genes that affect serum thyroid hormone levels and/or have effects on thyroid hormone-related physiological endpoints. These polymorphism studies are important for several reasons.

First, new insight can be obtained about the physiological function of thyroid hormone pathway genes. The hypothesis regarding a relative decrease in the contribution of D2 to serum T3 production (Figure 2), based on the different associations of D1 and D2 polymorphisms in younger and elder populations, is an example of this (21, 70). Also the role of D2 and TSH receptor activity in the development of insulin resistance was previously unknown (22, 64, 78).

Second, genetic variation is very important in the inter-individual variation in thyroid hormone bioactivity (6-9). It seems that each individual has a different

thyroid function set-point, and small variations around this set-point, even within the normal range, can already have important consequences, for example on body weight (12). This raises the possibility to better estimate an individual's set-point, based on his/hers genetic make-up of thyroid hormone pathway genes. The decision wether a patient with subclinical changes in thyroid parameters should be treated, might then be made on that individual patient's normal values.

In addition, the decision to treat patients with subclinical thyroid disease is based on the risk of such patients to develop complications, such as atrial fibrillation in subclinical hyperthyroidism. If the genetic profile makes a patient more vulnerable to develop atrial fibrillation (90), this might be an indication to initiate treatment in an earlier phase. It should be noted that these latter remarks are highly speculative, and that the observed associations first have to be replicated in other, independent populations.

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# *General Discussion*

# *Part B*

## Changes within the thyroid axis during the course of critical illness

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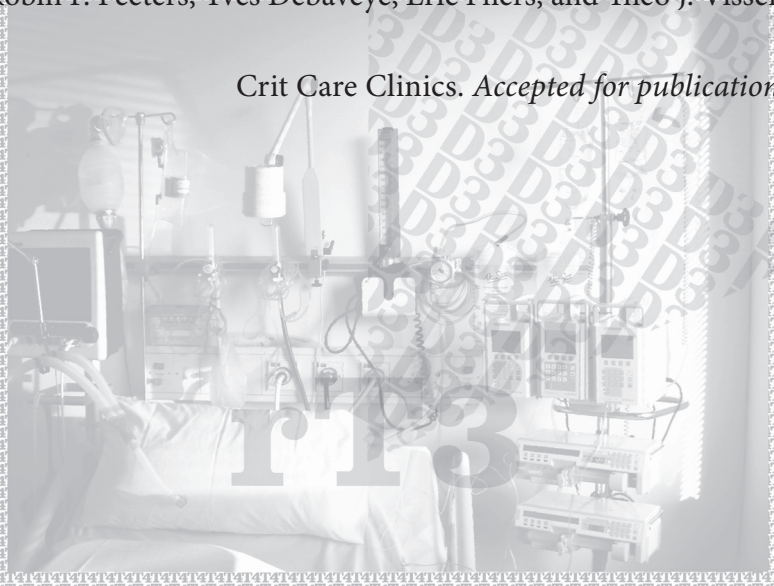

## General introduction

Thyroid hormone is essential for the regulation of energy metabolism, and for the physiological function of virtually all tissues (1, 2). The production of thyroxine (T4) by the thyroid gland is regulated by the classic hypothalamus-pituitary-thyroid axis, in which the anterior pituitary releases thyroid stimulating hormone (TSH, thyrotropin) under the stimulation of hypothalamic thyrotropin-releasing hormone (TRH) from the paraventricular nucleus (1-5). The biological activity of thyroid hormone, *i.e.*, the availability of the active hormone 3,5,3'-triiodothyronine (T3), is largely regulated by the iodothyronine deiodinases D1, D2 and D3 (1-3, 6), which convert T4 to either T3 or to the inactive metabolite reverse T3 (rT3). Both T4 and T3 have an inhibitory effect on TRH and TSH secretion via a negative feedback loop mechanism.

Within a few hours after the onset of disease, plasma T3 decreases and plasma rT3 increases, and the magnitude of these changes is related to the severity of the disease (6-9). Similar changes are observed after 24-36 hours of food deprivation, and disappear upon refeeding (7, 10). In severely ill patients, T4 decreases as well and both low T4 and low T3 are associated with a poor prognosis (7, 8, 11, 12). Although serum TSH levels may increase briefly after the onset of disease, circulating TSH usually remains within the normal range, despite the decreased levels of serum T3, and in severe illness also of serum T4 (7, 10, 13-16). The nocturnal surge of TSH that occurs in the normal physiological state is absent in the acute phase of critical illness (17, 18).

The thyrotropic profile in chronic critical illness differs from the acute phase. Patients who have been receiving intensive care for several days have even lower levels of T3 and a low T4 (12, 19, 20). In addition, levels of TSH are low to normal and there is hardly any pulsatility in the nocturnal TSH secretory pattern compared with healthy individuals (19), and hypothalamic expression of the TRH gene is low in patients who died after chronic severe illness compared to patients who died from an acute event (21). Obviously, not only the duration, but also the severity of illness is an important factor determining the changes in serum thyroid hormone levels.

Teleologically, the acute changes within the thyroid axis during starvation have been interpreted as an adaptation to reduce energy expenditure, which is beneficial and does not necessitate intervention (22, 23). Whether this also applies to the acute and especially the chronic phase of critical illness remains controversial, since it is hitherto unclear if the reduction in serum T3 is a beneficial adaptation that protects against hypercatabolism, or whether it is a mal-adaptation that contributes to a worsening of the disease (7, 13, 24). This review will discuss the mechanisms behind the observed changes,

focussing on the regulation of thyroid hormone deiodination and transport, as well as the potential positive and/or negative effects, both for the acute and the chronic phase of critical illness. For a discussion of changes in thyroid hormone receptor isoform expression during illness, the reader is referred to other papers (25-27).

## Hypothalamus-pituitary-thyroid-axis during critical illness

Although circulating TSH sharply increases in primary hypothyroidism, it remains within the normal range in critical illness, despite a major decrease in serum T3 and T4 levels (15, 28). This suggests that there is an altered feedback setting at the hypothalamic-pituitary level (29-32), which cannot be attributed to exogenous glucocorticoids or dopamine (19). Animal data show that a reduced expression of TRH, as well as an altered transmembrane transport and enhanced nuclear T3 receptor occupancy in the thyrotrophes might be involved (31, 33, 34). An increased expression of D2 in the hypothalamus, resulting in a higher local T3 production, may also play a role (35-38). The cytokines TNF-α, IL-1, and IL-6 might be involved in the pathogenesis, since they are (partially) capable of mimicking the thyrotropic alterations in the acute stress response (33, 39, 40). However, cytokine antagonists failed to restore thyroid hormone levels (41). Studies on IL-12 and IL-18 knockout mice show that these cytokines are also involved in the regulation of the hypothalus-pituitary-thyroid axis during illness (42, 43).

Patients receiving intensive care for a prolonged period of time show, in addition to the absent nocturnal TSH surge, a dramatically diminished TSH pulsatility, which is related to the low serum T3 levels (19, 32). Furthermore, patients who died after chronic severe illness have a low expression of TRH mRNA in the hypothalamic paraventricular nuclei compared to patients who died from an acute lethal trauma such as a road accident (21). Patients who die after severe illness have less than half the concentration of tissue T3 in hypothalamus and pituitary compared to patients who die acutely from trauma (44). This combination of low hypothalamic T3 and low TRH expression in the paraventricular nuclei implies a major change in the hypothalamic thyroid hormone feedback regulation during critical illness, and suggests that the decreased production of thyroid hormone in chronic severe illness has –at least in part- a central origin. The observation that TRH mRNA expression in the paraventricular nuclei is positively correlated with serum TSH and T3 levels in patients who died after chronic severe illness also points towards a major change in hypothalamic feedback regulation (21). Also the increased TSH as a marker for recovery supports this concept (12, 30, 45), as well as a post mortem study of the thyroid gland showing a diminished gland weight and follicular size in chronically

critically ill patients compared to patients who suffered an acute death (46). Furthermore, continuous infusion of TRH (especially combined with growth hormone secretagogues) in patients with prolonged critical illness can (partially) restore the serum concentrations of TSH, T4, and T3 in humans (32, 47-49).

The neuroendocrine pathogenesis behind these changes in the chronic phase of severe illness are not yet fully known. Since circulating cytokines are usually low in this phase (50), other mechanisms must be involved. Prolonged hypercortisolism during critical illness may play a role, since glucocorticoids decrease TRH secretion (13, 14, 51, 52). Immunocytochemical staining of neuropeptide Y (NPY) cells in the infundibular nucleus yielded very weak staining in patients with chronic illness compared with patients who had suffered an acute death, and this was associated with decreased TRH mRNA expression in the paraventricular nucleus (28). These data suggest that the hypothalamus plays an important role in the neuroendocrine response during critical illness (28, 53). An upregulation of D2 in the mediobasal hypothalamus, which is seen in rats after lipopolysaccharide injection, may also very well contribute to the suppressed hypothalamus-pituitary-thyroid axis via an increased local T3 production (37, 38). Another theoretical possibility leading to relatively high hypothalamic T3 concentrations during protracted illness is down-regulation of D3 in the paraventricular nucleus where D3 is expressed in the human hypothalamus (54). However, patients who died after severe illness had half the concentration of T3 in the hypothalamus compared to patients who had died from trauma (44). The expression of human MCT8 in a minority of the TRH neurons of the paraventricular nucleus, and also in other regions of the hypothalamus, suggests that regulation of MCT8 activity may also be important in the altered hypothalamic set point in critical illness (54). Also the possible regulation of the OATP1C1 high affinity T4 transporter in the brain (55), and of D3 and/or the different thyroid hormone receptor isoforms, which are abundantly expressed in the infundibular nucleus, might be involved (54, 56). In this context it is of interest that thyroid hormone receptor isoform expression was shown to be differentially regulated by thyroid hormone status in different brain regions including the PVN (57). These possibilities remain to be elucidated in future studies.

## Thyroid hormone metabolism during critical illness

While the changes in serum thyroid hormone levels in the chronic phase of severe illness are mainly of neuroendocrine origin, the peripheral metabolism of thyroid hormone seems to be the main player in the acute phase of severe illness (7, 10, 13, 29, 58). Especially the combination of decreased serum T3 and increased serum

rT3 levels, and thus a decreased T3/rT3 ratio, which occurs within a few hours after the onset of severe stress, suggests major changes in the peripheral metabolism of thyroid hormone. The serum T3/rT3 ratio is the parameter that most accurately reflects the result of altered peripheral thyroid hormone metabolism during critical illness, because of the confounding effect of variable concentrations of T4 and T4-binding proteins (12, 20, 59).

Thyroid hormone metabolism is mediated importantly by the three iodothyronine deiodinases D1, D2 and D3, that catalyze the inner and/or outer ring deiodination of the different iodothyronines (see Refs (3, 6) for reviews) (Fig. 1a). Outer ring deiodination is regarded as an activating pathway, whereas inner ring deiodination is an inactivating pathway. D1 is present in liver, kidney, and thyroid, and plays a key-role in the production of serum T3 from T4 and in the breakdown of the inactive metabolite rT3 (3, 6). D2 is present in brain, anterior pituitary, thyroid, and skeletal muscle, and D2 mRNA has been detected in the human heart. D2 also converts T4 by outer ring deiodination to T3. D2 is important for local T3 production in tissues such as the brain and pituitary, but the enzyme in skeletal muscle may also contribute to plasma T3 production (3, 6, 60). D3 is present in brain, skin, placenta, pregnant uterus, and various fetal tissues; it catalyzes the inactivation of T4 and T3 by inner ring deiodination to rT3 and 3,3'-diiodothyronine (3,3'-T2), respectively (3, 6). It is the major T3 and T4 inactivating enzyme since D1 has only weak inner ring deiodination capacity, and D2 has none. D3 contributes to thyroid homeostasis by protecting tissues from excess thyroid hormone.

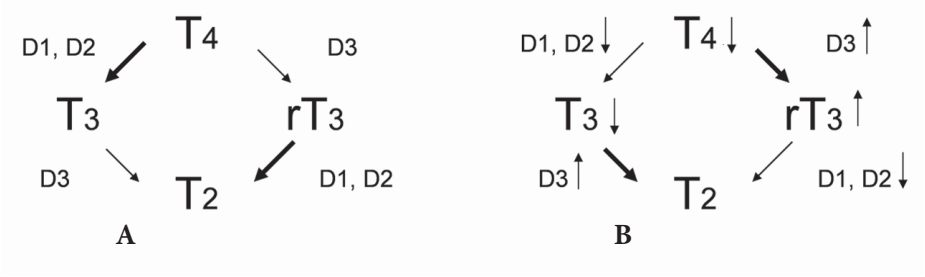


**Figure 1. A**) Relationship between the different iodothyronines and the outer (D1, D2) and inner ring (D3) deiodination by the three deiodinases. Like D3, D1 also has some inner ring deiodination activity *in vitro*, but there is currently no evidence that this is of any significance *in vivo*. This is therefore omitted from the figure. **B**) Observed changes in iodothyronine levels during critical illness, and the altered deiodinase activities contributing to these changes.

Studies on the role of deiodinases during critical illness have mainly focussed on D1, and only recently D2 has also been implied (6, 12, 20). The reduction in circulating T3 levels during critical illness was thought to be due to a decreased peripheral deiodination of T4 by D1, D2, or both (6, 61, 62). The increase in rT3 was explained by a decreased D1 activity, since D1 is the principal pathway for rT3 clearance (3, 6, 63). However, the reciprocal changes in T3 and rT3 can also be explained by an induction of D3, resulting in an increased degradation of T3 and an increased production of rT3. A high level of D3 expression in vascular tumors has been reported to result in very low levels of circulating T4 and T3 in some patients, combined with high levels of rT3 (64, 65). This condition is referred to as consumptive hypothyroidism.

In recent studies, a large number of post-mortem liver and skeletal muscle samples from critically ill patients who died in a surgical intensive care unit were analyzed (12, 20, 66, 67). Liver D1 activity in these patients was low compared to values observed in healthy individuals, except for patients who died acutely from severe brain damage (12, 20). Although D2 activity is expressed in normal skeletal muscle (68, 69), no D2 activity could be detected in skeletal muscle samples of 66 critically ill patients (12, 20, 67). On the other hand, D3, which is not present in liver and skeletal muscle in healthy individuals, was markedly induced in both liver and skeletal muscle (Fig. 1b) (12, 20, 67). In these critically ill patients, liver D1 activity was positively correlated with the serum T3/rT3 ratio, whereas liver and muscle D3 were positively correlated with serum rT3 levels, also in the group of patients who received intensive care for more than five days (Fig. 2) (12, 20). From these data it can be concluded that in addition to a down-regulation of D1, also a down-regulation of D2 and an induction of D3 expression are important in the regulation of serum thyroid hormone levels during critical illness. The lack of D2 activity in an abundant tissue as skeletal muscle, and the induction of D3 activity in liver and skeletal muscle, are likely to contribute to the low serum T3 and high serum rT3 levels observed in critically ill patients, and suggest that the role of D2 and D3 in thyroid hormone metabolism during critical illness was previously underestimated. D3 can decrease the T3/rT3 ratio by two ways: it prevents conversion of T4 to T3 by catalyzing the conversion of T4 to rT3 instead, and it also catalyzes the degradation of T3 to 3,3'-T2. These data also show that an altered peripheral metabolism of thyroid hormone is not only a major factor in the acute phase of severe illness, but also in protra cted critical illness, since tissue deiodinase activities were significantly correlated with serum levels in chronic critically ill patients (12).

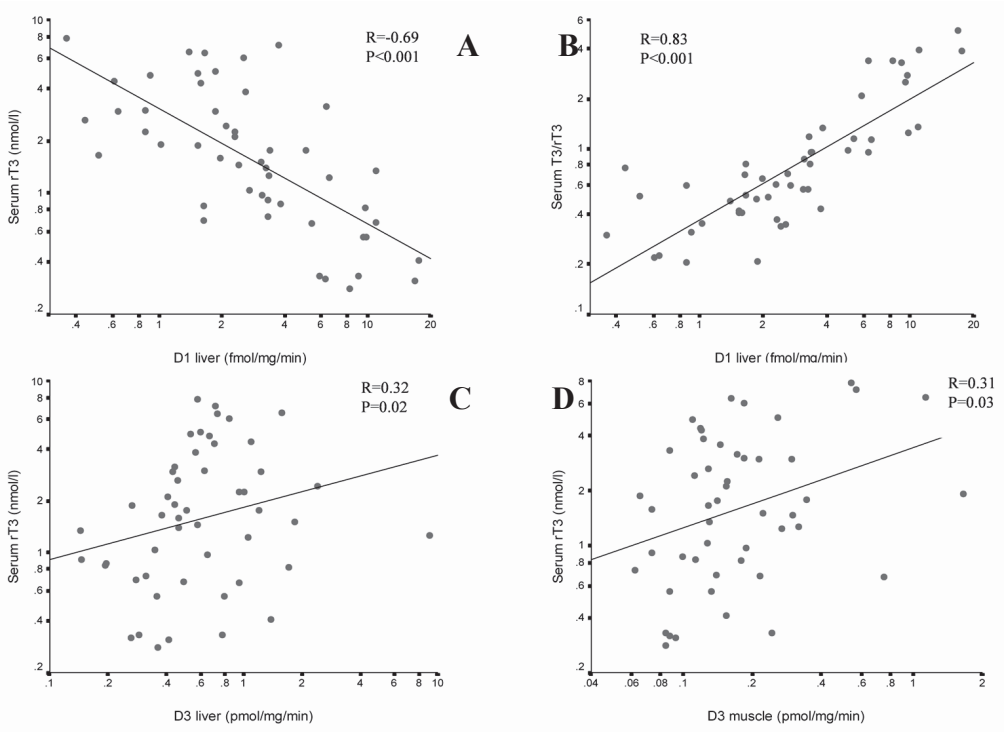


**Figure 2.** Correlation analysis of liver D1 activity and serum rT3 (A) and T3/rT3 (B), and of liver D3 activity (C) and skeletal muscle D3 activity (D) and serum rT3. Liver D1 showed a significant negative correlation with rT3 (P<0.001) and a significant positive correlation with T3/rT3 ratio (P<0.001). Both liver and skeletal muscle D3 activity were positively correlated to serum rT3 levels (P<0.05). R represents Spearman's correlation coefficient. To convert values for rT3 to ng/dL, multiply by 64.9.

Studies in 451 patients who received intensive care for at least five days, showed that serum rT3 and the T3/rT3 ratio on day 1 were already prognostic for survival, whereas TSH, T4 and T3 were significantly different between survivors and non-survivors from day 5 onward (12). Not only the absolute values, but also the time course was completely different between survivors and non-survivors. TSH, T4, T3, and the T3/rT3 ratio increased in patients who survived, whereas there was no such rise in patients who died. However, other than for TSH, T4, and T3, this increase in T3/rT3 in survivors occurred only after day 5, suggesting that the peripheral metabolism recovers at a later stage than the centrally initiated TSH secretion (12) (Fig. 3).

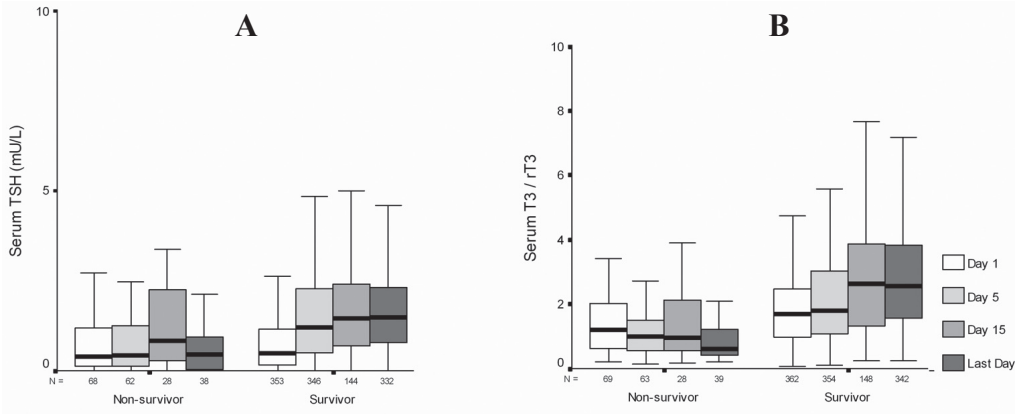


**Figure 3.** Serum TSH levels and the T3/rT3 ratio at day 1, 5, 15, and last day of ICU stay between survivors and non-survivors. From day 5 onward, serum TSH increased in patients who survived, whereas there was no such pattern in patients who died (Fig. 3A). The serum T3/rT3 ratio increased in survivors from day 5 to LD, whereas it did not alter or even decreased in non-survivors (Fig. 3B). Box plots represent 10th-25th-50th-75th-90th percentiles.To convert values for T4 to μg/dL, multiply by 0.0777, and to convert values for T3 or rT3 to ng/dL, multiply by 64.9.

Tissue concentrations were also analyzed in these critically ill patients (67). Liver D1 activity was negatively correlated with the rT3 concentration in liver, and positively with the liver T3/rT3 ratio in these patients (67). Tissue D3 activities were positively correlated with rT3 levels in both liver and skeletal muscle, and muscle D3 was negatively correlated with the T3/rT3 ratio (67). The complete absence of muscle D2 activity in these patients is likely to have resulted in a decreased T3 production in skeletal muscle (6, 68, 69). These data suggest not only an important role during critical illness for liver and skeletal muscle deiodinase activities in the regulation of serum concentrations of thyroid hormone, but also in the regulation of tissue thyroid hormone levels, and thus in the regulation of thyroid hormone bioactivity.

Sulfated iodothyronines do not bind to the thyroid hormone receptors, and sulfation mediates the rapid and irreversible degradation of iodothyronines by D1 (70). Therefore, the concentrations of sulfated iodothyronines in serum are normally low (71-74). Inner ring deiodination (inactivation) of T4 and T3 by D1 is markedly facilitated after sulfation, whereas outer ring deiodination of T4 is blocked after sulfation (75-77). D2 and D3 are incapable of catalyzing the deiodination of sulfated iodothyronines (77). Because of the decreased D1 expression during critical illness (6, 10, 12, 20), we recently analyzed the metabolism of iodothyronine sulfates in critically ill patients (66). T4S levels were increased in these critically ill patients, despite low levels of serum T4 (66). This could be due to an increased production

and/or a decreased clearance of T4S. Hepatic D1 activity showed a strong negative correlation with serum T4S and with the T4S/T4 ratio, whereas hepatic SULT activities were not correlated with these parameters (66). This suggests that the decreased activity of liver D1 during critical illness also plays an important role in the increase in serum T4S and T3S levels during critical illness.

## Transmembrane transport of thyroid hormone during critical illness

In addition to serum iodothyronine levels and tissue deiodinase expression, also transmembrane transport of iodothyronines is important in the regulation of thyroid hormone bioactivity. Inhibition of transport into hepatocytes leads to a diminished thyroid hormone metabolism, both in vitro and in vivo (78-81). Uptake of T4 by human hepatocytes is temperature, Na-, and energy-dependent (82), and kinetic analyses indicate that T4 and T3 cross the plasma membrane by different transporters (83, 84). There is evidence that a diminished transport of thyroid hormone into D1-expressing liver plays a role during critical illness (see refs (85, 86) for reviews). Apart from decreased levels of hepatic ATP during critical illness, increased plasma concentrations of compounds such as bilirubin and non-esterified fatty acids that inhibit transport, may play a role (87, 88). No data are currently available about possible differences in transport regulation in the acute versus the chronic phase of severe illness. A study using Xenopus oocytes as an expression system suggests little effect of thyroid state itself on the mRNA levels coding for T4 transporters in rat liver (89).

Recently, human MCT8 was identified as an active and specific thyroid hormone transporter (90), with a preference for T3 over T4 in humans (unpublished data). Although MCT8 transport activity is not Na- and/or energy-dependent, MCT8 is expressed in, among other tissues, liver and skeletal muscle (90). Studies on MCT8 mRNA expression in liver and muscle samples of critically ill patients suggest that MCT8 is not crucial in the transport of these iodothyronines over the plasma membrane in these tissues (67). This is supported by the fact that inactivating mutations in MCT8 result in elevated levels of T3, low levels of rT3, and a phenotype of severe mental retardation, but there is currently no evidence for liver or muscle hypothyroidism in these patients (91). The expression of human MCT8 in glial cells in the infundibular region and in neurons in the paraventricular nucleus and other hypothalamic nuclei expressing thyroid hormone receptors, suggests that regulation of MCT8 activity may be more important in the altered hypothalamic set point in critical illness (54). The role of MCT8, and of other putative thyroid hormone transporters during critical illness remain therefore to be elucidated in future studies.

## Thyroid hormone substitution during critical illness

Whether the reduction in serum T3 is an adaptation that results in a decreased metabolic rate and protects against hypercatabolism or whether it is a mal-adaptation that contributes to a worsening of the disease, is still controversial (7, 13, 24). When determining the possible role for thyroid hormone substitution in critically ill patients, it is important to realize the differences between the acute and the chronic phase of critical illness (13, 14, 29). The acute changes in the thyroid axis after the onset of critical illness are similar to those observed in fasting, and have therefore been interpreted as an attempt to reduce energy expenditure and protein wasting (22, 23). Carefully performed studies in fasting subjects suggest that replacement with thyroid hormone does result in increased catabolism, as indicated by the increased nitrogen excretion and negative nitrogen balance (23, 92-94). It is unclear whether this is also true for critical illness, but studies in rats show no beneficial or even negative effects of thyroid hormone treatment in critically ill rats (95, 96). So far, it has not been clearly demonstrated that substitution of critically ill patients with thyroid hormone has a positive or negative effect on clinical outcome (97-100) (101). Administration of T4 in patients with acute renal failure was associated with an increased mortality, which might have been due to the suppressed TSH in the treatment group (101). Free T4 and free T3 levels were not different between the two groups in this study (101). A randomized clinical trial, in which 11 patients who were admitted to an intensive care were treated with T4, showed no beneficial effect of T4 treatment on survival either (97). Nitrogen excretion was not analyzed in these studies. Although T4 levels in the treated patients rose into the normal range in the latter study, serum T3 concentrations remained low and did not differ between the two treatment groups (97). This might be due to the decreased T4 to T3 conversion by D1 and D2, which is seen in both the acute and chronic phase of critical illness, and by the accelerated breakdown of T4 and T3 by D3 (12, 20). Therefore, treatment with T3 may be a better choice, although also T3 may be detrimental and will also be subject of degradation by D3. More recent randomized clinical trials showed an improved cardiac function in adult patients treated with pharmacological doses of T3 for 6 hours after cross clamp removal during elective coronary artery bypass grafting (98), and in dopamine-treated children who received T3 substitution after cardiopulmonary bypass surgery (99). However, since there was no effect on (perioperative) survival, those results do not refute the postulated adaptive nature of the acute low T3 syndrome.

With the development of intensive care medicine in the last decades, patients who previously died from serious life-threatening diseases, can now survive. These

patients sometimes require a very long period of intensive care (several weeks). The changes in the thyrotropic profile that are seen in this situation seem to be of a more central origin (13, 14, 29), although there is also a decreased T4 to T3 conversion (12). Studies on fasting subjects should therefore not be extrapolated to this situation. In chronic critical illness, many tissues have reduced thyroid hormone levels, although the severity seems to vary from one organ to another (44). As in the acute situation, also in patients with protracted critical illness, low levels of thyroid hormone and a low T3/rT3 ratio are associated with a higher mortality rate (12). Furthermore, serum thyroid hormone levels are negatively correlated with urea production and bone degradation, which are markers of catabolism (13, 47). This suggests that the low levels of T4 and T3 in protracted critical illness reflect the severity of disease, and that it could be either adaptive, protecting against hypercatabolism or, alternatively, a maladaptation that contributes to the worsening of the disease which should be treated.

If we decide to treat these patients, the major question is with what should we treat them? And should we aim for thyroid hormone levels within or still below the normal range?

Because of the decreased T4 to T3 conversion by D1 and D2, which is also seen in the chronic phase of critical illness (12), T4 treatment does not seem appropriate and T3 treatment may be a better choice. A recent study, in which patients were treated with a combination of T4 and T3, showed tissue specific effects of thyroid hormone treatment in different tissues, with a disproportional increase in liver T3 levels compared to serum and skeletal muscle T3 concentrations (67). This is in line with studies in thyroidectomized rats, in which neither T4 nor T3 were able to restore euthyroidism in all tissues and plasma (102-104).

A major disadvantage of substitution with thyroid hormone itself, is that the hypothalamus-pituitary-thyroid axis is bypassed. This may result in over-treatment and TSH suppression, whereas an increase in serum TSH marks the onset of recovery and concomitantly drives the increase in serum T4 (7, 12, 30, 45). If a combination of T4 and T3 (or T3 alone) is given, the local regulation of thyroid hormone bioactivity by T4 to T3 conversion is also bypassed, which may be an additional argument against giving a combination of T4 and T3. Intervention with hypothalamic releasing factors might therefore be a more successful approach (47, 49, 105). Continuous infusion of TRH, combined with a growth hormone secretagogue, restored pulsatile pituitary hormone secretion, restored physiological levels of thyroid hormone and reduced urea production and bone degradation as markers of catabolism in a group of patients with prolonged critical illness (47, 49). Whether this

treatment also results in an improved clinical outcome in patients with protracted critical illness, remains to be determined in a randomized clinical trial.

## Summary

Pronounced alterations in plasma TSH and thyroid hormone levels occur during critical illness without any evidence for thyroid disease. Plasma T3 decreases and plasma rT3 increases within a few hours after the onset of disease, and the magnitude of these changes is related to the severity and the duration of the disease. Here we reviewed the mechanisms behind the observed changes, focussing on the regulation of thyroid hormone deiodination and transport, as well as the potential positive and/or negative effects, both for the acute and the chronic phase of critical illness.

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Summary

## Summary and conclusions

Thyroid hormone plays an essential role in a variety of metabolic processes in the human body. Examples are the effects of thyroid hormone on metabolism and on the heart. The production of thyroid hormone by the thyroid is regulated by thyroid stimulating hormone (TSH) via the TSH receptor. The thyroid produces T4, which is not biologically active. Therefore, T4 has to be converted to the active hormone T3, a process that is regulated by three enzymes, the deiodinases (D1-D3). D1 and D2 are thyroid hormone activating enzymes that catalyze the conversion of T4 to T3. D3 is an inactivating enzyme that converts T4 to the inactive metabolite rT3 and breaks down T3 to 3,3'-T2. In the *General Introduction*, a more detailed overview of the regulation of thyroid hormone production, as well as the regulation of thyroid hormone bioactivity, is presented. Furthermore, the rationale behind the performed studies and an outline of the thesis are presented.

This PhD project has focused on the regulation of thyroid hormone bioactivity, and consists of two major parts. In **Part A** of the thesis, we have analyzed the importance of subtle genetic variations in thyroid hormone pathway genes. Current estimates are that ~65% of inter-individual variation in serum thyroid hormone levels is due to genetic factors. Already minor changes in thyroid hormone levels can have important effects on metabolism and heart rate. We therefore investigated genes encoding key proteins in thyroid hormone metabolism for the occurrence of genetic variations (polymorphisms). Since these polymorphisms may have subtle effects on thyroid hormone bioactivity throughout life, we also analyzed if they were associated with thyroid hormone-related clinical endpoints.

In *Chapter A1*, we have analyzed several target genes in thyroid hormone production or metabolism for the possible occurrence of polymorphisms. We identified several new polymorphisms in the deiodinases and the TSH receptor (TSHR). Some of these polymorphisms were associated with serum thyroid parameters in a population of healthy blood donors, suggesting that they are functionally relevant. Regarding two polymorphisms located in the D1 3'-untranslated region (UTR), the T-allele of D1a-C785T was associated with a lower T3/rT3 ratio, whereas the G-allele of D1b-A1814G was associated with a higher T3/rT3 ratio. Based on the function of D1, it is likely that D1a-C>T results in a decreased activity of D1, whereas D1b-A>G results in an increased activity. The Glu-allele of TSHR polymorphism Asp727Glu was associated with lower levels of plasma TSH, but similar levels of FT4. This suggests that the Glu-allele variant of TSHR has a higher activity than the wild-type receptor, since less TSH would be required to achieve a normal thyroid hormone production.

There is a close interaction between growth hormone and thyroid hormone metabolism, which is complex and not fully understood. In *Chapter A2* we therefore analyzed the association of the D1 polymorphisms with insulin-like growth factor –I (IGF-I) levels. The D1a-785T allele was associated with increased levels of free IGF-I in two unrelated populations, which was substantiated by the association of this haplotype allele with several IGF-I related endpoints, such as increased muscle strength and muscle mass. Furthermore, the D1a-785T allele was associated with lower levels of T3 in elderly men, which supported the theory of a lower D1 activity in carriers of this polymorphism.

In *Chapter A3* we identified a new polymorphism, located in a short open reading frame (ORFa) in the 5'UTR of the D2 mRNA. This ORFa has been shown to be an important regulatory element, and mutations in the start codon of ORFa, have enormous consequences for D2 activity in vitro. The Asp-allele of this polymorphism (D2-ORFa-Gly3Asp) is associated with an increased serum T3/T4 ratio in younger, but not in elderly subjects, supporting our hypothesis of a relative decrease in the contribution of D2 to serum T3 production with an increase in age (Figure 2 from general discussion A).

Polymorphisms in D1 are associated with body composition (*Chapter A2*), and thyroid hormone is essential for the regulation of energy metabolism. In *Chapter A4* we therefore analyzed the association of polymorphisms in D2 and in the TSHR with insulin resistance. In a population of relatively healthy elderly men, we showed that polymorphisms in D2 are associated with insulin resistance, and we demonstrated that the TSHR-Asp727Glu polymorphism is twice as frequent in subjects with diabetes as in subjects without diabetes. Furthermore, in the non-diabetic subjects this TSHR polymorphism was associated with in an increased fat mass and in a 50% increase in HOMA, which is a measure of insulin resistance. Based on these and other polymorphisms, it might eventually be possible to better estimate a subject's risk of developing diabetes, and this may be a reason to check some people more regularly than others.

Patients with (subclinical) hyperthyroidism have an increased risk of atrial fibrillation, which is an important cardiac arrhythmia. Overexpression of D2 in the mouse heart results in an increased heart rate. In *Chapter A5* we therefore analyzed whether polymorphisms affecting D2 activity also result in an altered heart rate and risk of atrial fibrillation. We demonstrated that subjects with a genetically determined higher D2 activity have a 3 times higher risk of developing atrial fibrillation. Since atrial fibrillation can be lethal, it is very important to be able to identify the patients who have a high risk of developing this serious condition.

In **Part A** of the *General Discussion*, we discuss the importance of the polymorphism studies we have performed. These studies are important for several reasons. Not only can new insights be obtained in the physiological function of thyroid hormone pathway genes, but genetic variation is also very important in the inter-individual variation in thyroid hormone bioactivity. Each individual seems to have a different thyroid function set-point, and small variations, even within the normal range, around this setpoint have important consequences for example for body weight. In the future it might become possible to better estimate an individual's set-point, based on his/hers genetic variation in thyroid hormone pathway genes. The decision whether a patient with subclinical changes in thyroid hormone levels should be treated, might then be made on that individual patient's setpoint. In addition, the decision to treat patients with subclinical thyroid disease is based on the risk of that patient to develop complications, such as atrial fibrillation or insulin resistance. If the genetic profile makes a patient more vulnerable to develop such complications, this might be an indication to initiate treatment in an earlier phase.

In **Part B** of this thesis, we have focused on the regulation of thyroid hormone bioactivity during critical illness. Pronounced alterations in TSH and thyroid hormone levels occur during critical illness, without any evidence for thyroid disease. These changes result in low serum levels of bioactive T3 and in high levels of the inactive metabolite rT3. Whether this is a beneficial adaptation or that it is a mal-adaptation, contributing to a worsening of the disease, is still controversial. We therefore investigated the mechanisms behind these changes in a large group of critically ill patients, and we investigated whether these changes are associated with the prognosis of the patient.

In *Chapter B1* we analyzed serum, liver and skeletal muscle samples of 80 critically ill patients who died in an intensive care unit. We demonstrated that the thyroid hormone activating enzymes D1 and D2 were markedly reduced or even undetectable, whereas the thyroid hormone inactivating enzyme D3, which is not present in liver or muscle of healthy subjects, was induced. There was a good correlation between enzyme activities, mRNA levels and protein data. Serum thyroid hormone levels in these patients were significantly correlated with the tissue deiodinase activities.

In *Chapter B2* we investigated whether serum TSH and iodothyronine levels are prognostic factors for survival. In 451 patients with prolonged critical illness (>5 days) we determined serum thyroid parameters on different days of intensive care, and found that patients with high rT3 levels and a low active over inactive hormone ratio (T3/rT3) had an increased chance of dying. From day 5 onward, TSH, T4, T3 levels, and also the T3/rT3 ratio increased in patients who would survive, but not

in the non-survivors. Again, serum thyroid hormone levels in these patients were significantly correlated with the tissue deiodinase activities.

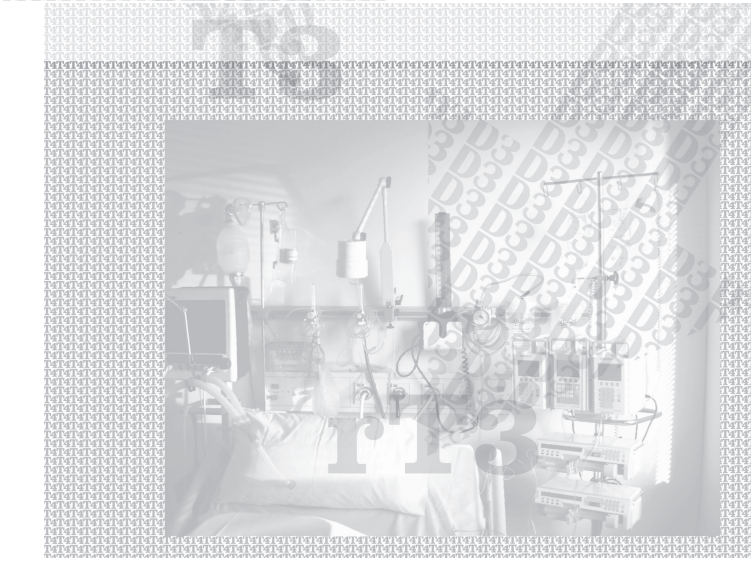
Since we had shown a decreased expression of D1 during critical illness, and since D1 mediates the rapid and irreversible degradation of iodothyronine sulfates, we analyzed the metabolism of iodothyronine sulfates during critical illness in *Chapter B3*. Serum levels of T4S were clearly elevated compared to healthy references, which seemed to be due to a decreased activity of liver D1. D1 is known to deiodinate T4S very rapidly to rT3S but conversion to T3S is blocked. Hepatic D1 activity was negatively correlated with serum T4S and with the T4S/T4 ratio, whereas hepatic sulfotransferase activities were not.

Since it is unclear if the changes in serum thyroid hormone levels are also reflected by changes in tissue concentrations, we investigated the regulation of local iodothyronine concentrations in liver and skeletal muscle in *Chapter B4.* Our data of 79 patients demonstrated that the low serum T4 and T3 concentrations during critical illness are accompanied by low levels of tissue T4 and T3 in liver and muscle. These local thyroid hormone levels were correlated with tissue deiodinase activities. Part of the studied patients had been treated with thyroid hormone. Those patients had higher levels of liver T3, and higher levels of muscle T4 and T3, suggesting that the effect of thyroid hormone treatment is tissue specific.

In addition (*Chapter B5, addendum*), we analyzed cortisol metabolism in these patients, since critical illness results in increased levels of cortisol, which might be accompanied by a peripheral adaptation in glucocorticoid sensitivity. We demonstrated a negative relation of different glucocorticoid receptor (GR) splice variants with circulating levels of cortisol and severity of illness, suggesting a down-regulation of GR expression in critical illness. Liver, but not muscle GR expression was substantially lower in patients who received exogenous glucocorticoids, which may make muscle tissue of these patients more vulnerable to the negative side effects of glucocorticoid treatment.

In Part B of the *General Discussion*, we discuss the importance of the studies we have performed in the criticaly ill patients. Never before has anyone studied the relation of deiodinase activities, and serum and tissue thyroid parameters in the human situation. Our data show that dramatic changes in the peripheral metabolism of thyroid hormone occur during critical illness. They also demonstrate that the more severe these changes are, the worse the prognosis of the patient, and that thyroid hormone treatment in these patients results in a local increase in thyroid hormone in liver and muscle. Whether thyroid hormone treatment also affects the prognosis of these patients, will be the subject of future studies.

# *Samenvatting*



# Samenvatting en conclusies

Schildklierhormoon speelt een essentiële rol in verscheidene metabole processen in het menselijk lichaam. Voorbeelden hiervan zijn het effect van schildklierhormoon op het basaalmetabolisme, op de lichaamssamenstelling, en op het hart ritme. De produktie van schildklierhormoon door de schildklier (thyroid) wordt gestimuleerd door TSH (Thyroid Stimulating Hormone) via de TSH receptor. De schildklier produceert vooral T4, wat zelf niet biologisch actief is. T4 moet daarom worden omgezet in het actieve hormoon T3, een proces dat gereguleerd wordt door drie enzymen, de zogeheten dejodases (D1, D2, en D3). D1 en D2 zijn schildklierhormoon activerende enzymen, die T4 naar T3 omzetten. D3 is een inactiverend enzym, dat T3 afbreekt en T4 omzet naar de inactieve metaboliet rT3. In de algemene introductie van dit proefschrift wordt meer gedetailleerd beschreven hoe de productie en activiteit van schildklierhormoon geregeld wordt. Ook wordt een overzicht van dit proefschrift gepresenteerd en wordt besproken waarom we de verschillende studies hebben gedaan.

Dit proefschrift over de regulatie van de bioactiviteit van schildklierhormoon en bestaat uit twee delen. In **deel A** hebben we onderzocht wat de gevolgen zijn van subtiele variaties in het DNA van genen die betrokken zijn bij het metabolisme van schildklierhormoon. Tussen verschillende personen bestaat er een grote variatie in schildklierhormoonconcentraties, terwijl de concentratie van schildklierhormoon in het bloed van een individu redelijk constant is. Ongeveer 65% van deze variatie tussen personen is te verklaren door variatie in het DNA. Minimale verschillen in schildklierhormoonconcentraties kunnen al belangrijke gevolgen hebben voor bijvoorbeeld het basaalmetabolisme, de lichaamssamenstelling en op de hartslag. Om deze reden hebben we het DNA dat codeert voor eiwitten die belangrijk zijn bij het metabolisme van schildklierhormoon onderzocht op de aanwezigheid van genetische variaties (polymorfismen). Omdat deze polymorpfismen effecten kunnen hebben op de activiteit van schildklierhormoon gedurende het hele leven, hebben we ook onderzocht of personen met of zonder bepaalde polymorfismen vaker bepaalde, schildklierhormoon gerelateerde aandoeningen hebben.

In *hoofdstuk A1* hebben we het DNA dat codeert voor eiwitten die betrokken zijn bij de produktie en het metabolisme van schildklierhormoon onderzocht op de aanwezigheid van polymorfismen. In zowel de dejodases als de TSH receptor hebben we verschillende nieuwe polymorfismen geïdentificeerd. In een groep gezonde bloeddonoren bleek een aantal polymorfismen geassocieerd te zijn met

schildklierhormoon concentraties in het bloed. Dit suggereert dat de betreffende variaties functioneel relevant zijn. Een variant in type I dejodase, D1-C785T (dit houdt in dat personen met deze variant op positie 785 van het mRNA van D1 een T in plaats van een C hebben) was geassocieerd met een lagere serum T3/rT3 ratio. Gezien de functie van D1 (produktie van T3 en afbraak van rT3) is het waarschijnlijk dat D1-C785T resulteert in een verminderde activiteit van D1. In een andere, veel oudere populatie, bleek D1-C785T geassocieerd te zijn met lagere waarden van serum T3 (*hoofdstuk A2*). Dit gaf steun aan de hypothese dat dit polymorfisme resulteert in een lagere activiteit van D1. Een tweede polymorfisme in D1, D1-A1814G, was geassocieerd met een hogere serum ratio, en dus waarschijnlijk met een hogere D1 activiteit. Een polymorfisme in de TSH receptor (Asp727Glu) was geassocieerd met lagere waarden van het plasma TSH, maar met een gelijk niveau van T4. Dit suggereert dat de variant receptor een verhoogde respons heeft op TSH, omdat er bij minder TSH toch een normale produktie van schildklierhormoon is.

Er is een complexe interactie tussen tussen het metabolisme van groeihormoon en schildklierhormoon, welke nog niet geheel opgehelderd is. Een groot deel van de effecten van groeihormoon wordt gemedieerd door de op insuline gelijkende groeifactor (IGF-I). In *hoofdstuk A2* hebben we daarom gekeken naar de relatie tussen polymorfismen in D1 en IGF-I concentraties. Het D1-C785T polymorfisme was in twee verschillende populaties geassocieerd met hogere concentraties van IGF-I. Het belang van deze associatie werd benadrukt doordat dit polymorfisme ook geassocieerd was met verschillende eindpunten die wijzen op een hogere activiteit van IGF-I, zoals spierkracht en spiermassa.

In *hoofstuk A3* hebben we een nieuw polymorfisme geidentificeerd in D2. Dit polymorfisme ligt in een zogenoemd Open Reading Frame (ORFa) van het D2 gen. Van dit ORFa is aangetoond dat het belangrijk is voor de regulatie van D2 activiteit. Het gevonden polymorfisme (D2-ORFa-Gly3Asp) bleek in jonge, maar niet in oudere mensen geassocieerd te zijn met een hogere serum T3/T4 ratio. Omdat D2 T3 produceert uit T4, suggereert deze associatie dat het polymorfisme resulteert in een hogere activiteit van D2. Bovendien suggereren deze data ook dat de bijdrage van D2 aan de produktie van serum T3 relatief minder wordt bij een toename van de leeftijd (zie Figuur 2 van de *algemene discussie*). Dit kan verklaard worden doordat met name het D2 in spierweefsel verantwoordelijk is voor de bijdrage aan de serum T3 productie, en met een toename van de leeftijd is er een duidelijk afname van zowel de kracht als van de hoeveelheid spierweefsel.

Schildklierhormoon is essentieel voor de regulatie van het energie metabolisme, en polymorphismen in D1 zijn geassocieerd met lichaamssamenstelling (*hoofdstuk*

*A2*). Gezien de belangrijke rol die het basaalmetabolisme en lichaamssamenstelling spelen bij de ontwikkeling van type 2 diabetes (insuline resistentie), hebben we in *hoofdstuk A4* onderzocht of polymorfismen in de dejodasen en in de TSH receptor geassocieerd zijn met insuline resistentie. We hebben daarbij aangetoond dat polymorfismen in zowel D2 als in de TSH receptor geassocieerd zijn met insuline resistentie. Bovendien komt het TSHR-Asp727Glu polymorfisme twee keer zo vaak voor in oudere patienten met diabetes als in ouderen zonder diabetes. Ook was dit polymorfisme in gezonde ouderen geassocieerd met een toename in lichaamsvet, en met een toename van 50% in de HOMA, wat een maat is voor (relatieve) insuline resistentie. Gebaseerd op deze (en andere) polymorfismen zal het in de toekomst mogelijk worden om een betere inschatting te kunnen maken van iemands risico op het ontwikkelen van diabetes. Dit kan dan een reden zijn om sommige patienten vaker te controleren dan anderen.

Patienten met subtiel verhoogde concentraties van schildklierhormoon (subklinische hyperthyreoidie) hebben een verhoogd risico op het ontwikkelen van een belangrijke hartritme stoornis, genaamd atrium fibrillatie. Een verhoogde expressie van het schildklierhormoon activerende D2 in resulteert in proefdieren in een toegenomen hartslag. Om deze reden hebben we in *hoofstuk A5* onderzocht of polymorfismen die resulteren in een veranderde acitiviteit van D2, ook geassocieerd zijn met een veranderde hartslag en/of met een verhoogd risico op het ontwikkelen van atrium fibrillatie. We hebben aangetoond dat personen die op basis van hun DNA een hogere of lagere activiteit van D2 hebben, een 3x hogere dan wel lagere kans hebben op het ontwikkelen van atrium fibrillatie. Het is erg belangrijk om patienten met een hoog risico op deze aandoening te kunnen identificeren, omdat atrium fibrillatie dodelijk kan zijn maar relatief makkelijk te behandelen is.

In deel A van de *algemene discussie* bespreken we het belang van de studies die we hebben gedaan naar de genetische variatie. Om een aantal redenenzijn deze studies belangrijk. Allereerst worden op deze manier nieuwe inzichten verkregen over de fysiologische rol van genen die betrokken zijn bij de regulatie van schildklierhormoon activiteit. Bovendien is genetische variatie een belangrijke oorzaak van de variaties in schildklierhormoon concentraties tussen verschillende personen. Ieder individu lijkt als het ware zijn eigen normaalwaarden te hebben voor schildklierhormoon. Kleine variaties rondom dit set-point, zelfs wanneer ze nog binnen de normale spreiding in de populatie zijn, kunnen belangrijke gevolgen hebben voor bijvoorbeeld lichaamsgewicht en het ontwikklen van diabetes. In de toekomst moet het mogelijk kunnen worden om op basis van de variatie in schildklierhormoon gerelateerde genen een inschatting te kunnen

maken van iemands setpoint. De beslissing of een patient met subklinische schildklierafwijkingen behandeld moet worden, kan dan gemaakt worden op basis van het setpoint van dat individu. Bovendien is de beslissing om een patient met subklinische schildklierafwijkingen wel of niet te behandelen afhankelijk van het risico dat die patient heeft op het ontwikkelen van complicaties, zoals bijvoorbeeld insuline resistentie en atrium fibrillatie. Als bepaalde polymorfismen een patient kwetsbaarder maken om dergelijke complicaties te ontwikkelen, kan dit een indicatie zijn om eerder te starten met behandeling.

In deel B van dit proefschrift hebben we ons gericht op de regulatie van schildklierhormoon bioactiviteit tijdens ernstige ziekte. Tijdens ernstige ziekte treden uitgesproken veranderingen op in zowel het TSH als in schildklierhormoonspiegels, zonder dat er sprake is van duidelijke ziekte van de schildklier zelf. Deze veranderingen resulteren in lage serumwaarden van het actieve hormoon T3 en in hoge waarden van de inactieve metaboliet rT3. Of dit een gunstige aanpassing is, of dat er juist sprake is van een maladaptatie die bijdraagt aan een verslechtering van de ziekte, is nog altijd controversieel. Om die reden hebben wij in een grote groep ernstig zieke patienten onderzocht wat de mechanismen zijn achter deze veranderingen, en ook hebben we onderzocht of deze veranderingen gerelateerd zijn aan de prognose van de patient.

In *hoofdstuk B1* hebben we serum, en lever- en spierbiopten onderzocht van 80 patienten die zijn overleden op een intensive care unit. We hebben aangetoond dat de schildklierhormoon activerende enzymen D1 en D2 duidelijk gereduceerd of zelfs geheel afwezig waren. Aan de andere kant was het schildklierhormoon inactiverende D3, wat bij gezonde personen in de lever en spier niet meetbaar is, juist geinduceerd bij deze ernstig zieke patienten. Er was een goede correlatie tussen enzym activiteit en de mRNA en eiwit niveaus van de dejodases. Tevens was er een duidelijke relatie tussen de weefsel dejodase activiteit en de serum schildklierhormoon concentraties.

In *hoofdstuk B2* hebben we onderzocht of serum TSH en schildklierhormoon concentraties tijdens ernstige ziekte een voorspellende waarde hebben voor overleving. In 451 patienten met chronisch ernstige ziekte (> 5 dagen) hebben we serum schildklierhormoon waarden gemeten op verschillende tijdstippen. Daarbij toonden we aan dat patienten met een hoog rT3 gehalte en met een lage ratio van het actieve over het inactive hormoon (T3/rT3) een verhoogde kans hadden om te overlijden. Vanaf dag 5 gingen het TSH, T4, T3, en ook de T3/rT3 ratio omhoog in de patienten die zouden overleven, maar niet in de patienten die uiteindelijk overleden. Ook in deze patienten was er een duidelijke relatie van de weefsel dejodase activiteit met de serum schildklierhormoon concentraties.

Omdat we hebben aangetoond dat D1 duidelijk verlaagd is tijdens ernstige ziekte, en omdat D1 verantwoordelijk is voor de snelle en irreversibele afbraak van schildklierhormoon sulfaat, hebben we in *hoofdstuk B3* het metabolisme van schildklierhormoon sulfaat onderzocht. De serum concentratie van T4 sulfaat (wat niet meer geactiveerd kan worden naar T3) was duidelijk verhoogd ten opzichte van gezonde referenties, wat met name leek te komen door de verminderde activiteit van D1 in de lever. Er was een negatieve relatie tussen D1 activiteit en de serum T4 sulfaat concentratie, terwijl verschillende sulfotransferases in de lever geen enkele relatie toonden met het T4 sulfaat gehalte in het bloed.

Omdat het niet duidelijk is of de veranderingen in schildklierhormoon concentraties in het bloed ook resulteren in veranderde concentraties in de weefsels, hebben we in *hoofdstuk B4* de regulatie van de lokale schildklierhormoon concentraties in zowel de lever als de spier onderzocht. Analyse van 79 patienten toonde aan dat de lage T4 en T3 concentraties in het bloed ook daadwerkelijk gepaard gaan met lage T4 en T3 concentraties in de lever en de spier. Ook was er een duidelijke relatie tussen deze lokale concentraties en de dejodase activiteit in het betreffende weefsel. Een deel van deze groep patienten was behandeld met schildklierhormoon. Deze behandelde patienten hadden hogere niveaus van weefsel T3 dan onbehandelde patienten, en verschillen tussen lever en spier suggereerden dat de effecten van schildklierhormoon behandeling weefselspecifiek zijn.

Tenslotte hebben we in het aanvullende *hoofstuk B5* ook het metabolisme van cortisol (=stress hormoon) geanalyseerd tijdens ernstige ziekte. Ernstige ziekte resulteert namelijk in verhoogde concentraties van cortisol. Dit zou gepaard kunnen gaan met een aanpassing in cortisol gevoeligheid van verschillende weefsels. We toonden aan dat een verhoogd serum cortisol en een ernstige ziekte gepaard gaan met een verlaagde expressie van de verschillende cortisol receptor varianten. Dit suggereert dat de expressie van de cortisol receptor naar beneden gaat tijdens ernstige ziekte. De expressie van deze receptor was in de lever, maar niet in de spier verlaagd bij patienten die werden behandeld met steroiden. Dit zou de spieren van deze patienten vatbaarder maken voor de negatieve bijwerkingen van steroid behandeling.

In deel B van de *algemene discussie* bespreken we het belang van de studies die we hebben uitgevoerd in ernstig zieke patienten. Nooit eerder heeft iemand in een dergelijke klinische setting gekeken naar de relatie van dejodase activiteit, en serum en weefsel concentraties van schildklierhormoon. Onze data tonen dat er dramatische veranderingen plaatsvinden in het perifere metabolisme van schildklierhormoon tijdens ernstige ziekte. Ook tonen we aan dat de prognose van patienten slechter is naarmate de veranderingen meer uitgesproken zijn,

en dat behandeling met schildklierhormoon in deze patienten resulteert in een lokale stijging van de schildklierhormoonconcentratie in zowel lever als spier. Of behandeling met schildklierhormoon ook daadwerkelijk resulteert in een verbeterde prognose van deze patienten, zal het onderwerp zijn van toekomstige studies.

# *Dankwoord*

# Dankwoord

Na drie jaar onderzoek en anderhalf jaar klinische opleiding is het dan nu zover dat ik kan beginnen aan het meest gelezen deel van ieder proefschrift. De afgelopen jaren zijn voor mij in veel opzichten een verrijking geweest. Niet alleen heb ik op wetenschappelijk en medisch inhoudelijk gebied enorm veel geleerd, ook voor mijn persoonlijke ontwikkeling was deze periode zeer waardevol. Zoals iedereen weet komt een promotie nooit in je eentje tot stand, en bij deze wil ik dan ook iedereen bedanken die heeft bijgedragen aan het tot stand komen van dit boekje. Een aantal mensen wil ik in het bijzonder noemen;

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# List of publications

# &

# Curriculum Vitae

# List of publications:

## Publications based on the studies described in this thesis

### *Part A*

I **RP Peeters, H van Toor, W Klootwijk, YB de Rijke, GG Kuiper, AG Uitterlinden, TJ Visser** 2003 Polymorphisms in thyroid hormone pathway genes are associated with plasma TSH and iodothyronine levels in healthy subjects. J Clin Endocrinol Metab. 88:2880-8

II **RP Peeters, AW van den Beld, H van Toor, AG Uitterlinden, JA Janssen, SW Lamberts, TJ Visser** 2005 A polymorphism in type I deiodinase (D1) is associated with circulating free IGF-I levels and body composition in humans. J Clin Endocrinol Metab. 90:256-63

III **RP Peeters, AW van den Beld, H Attalki, H van Toor, YB de Rijke, GG Kuiper, SW Lamberts, JA Janssen, AG Uitterlinden, TJ Visser** 2005 A new polymorphism in the type 2 deiodinase (D2) gene is associated with serum thyroid parameters. Am J Physiol Endocrinol Metab. 289:E75-81

IV **RP Peeters, AW van den Beld, H van Toor, SW Lamberts, JA Janssen, AG Uitterlinden, TJ Visser** 2005 Additive effects of the Asp727Glu polymorphism in the TSH receptor and the Thr92Ala polymorphism in the type II deiodinase on insulin resistance. S*ubmitted*

V **RP Peeters, FJ de Jong, YB de Rijke, AG Uitterlinden, A Hofman, MM Breteler, TJ Visser** 2005 Polymorphisms in the type II deiodinase are associated with atrial fibrillation in elderly subjects. *Manuscript in preparation*

### *Part B*

I **RP Peeters, PJ Wouters, E Kaptein, H van Toor, TJ Visser, G Van den Berghe** 2003 Reduced activation and increased inactivation of thyroid hormone in tissues of critically ill patients. J Clin Endocrinol Metab. 88:3202-11

II **RP Peeters, PJ Wouters, E Kaptein, H van Toor, TJ Visser, G Van den Berghe** 2005 Serum rT3 and T3/rT3 are prognostic markers in critically ill patients and are associated with postmortem tissue deiodinase activities. J Clin Endocrinol Metab. 90:4559-65

III **RP Peeters, MH Kester, PJ Wouters, E Kaptein, H van Toor, TJ Visser, G Van den Berghe** 2005 Increased T4S levels in critically ill patients due to a decreased hepatic type I deiodinase activity. J Clin Endocrinol Metab. Sep 27; [Epub ahead of print]

IV **RP Peeters, S van der Geyten, PJ Wouters, VM Darras, H van Toor, E Kaptein, TJ Visser, G Van den Berghe** 2005 Tissue thyroid hormone levels in critical illness. J Clin Endocrinol Metab. Sep 20; [Epub ahead of print]

V **RP Peeters, A Hagendorf, I Vanhorebeek, W Klootwijk, D Mesotten, PJ Wouters, JW Koper, FH de Jong, RA Feelders, TJ Visser, SW Lamberts, G Van den Berghe** 2005 Expression of the glucocorticoid receptor variants α, β, and P during critical illness. *Submitted*

### *General Discussion*

A **RP Peeters, W van der Deure, TJ Visser** 2005 Genetic variation in thyroid hormone pathway genes. *Submitted*

B **RP Peeters, Y Debaveye, E Fliers, TJ Visser** 2005 Changes within the thyroid axis during the course of critical illness. Crit Care Clinics *Accepted for publication*

## Other scientific publications

**J Pachucki, J Hopkins, R Peeters, H Tu, ED Abel, HC Kaulbach, FE Wondisford, J Ingwall, PR Larsen** 2001 Type 2 iodothyronine deiodinase transgene expression in the mouse heart causes cardiac-specific thyrotoxicosis. Endocrinology 142:13-20

**R Peeters, C Fekete, C Goncalves, HM Tu, JW Harney, AC Bianco, RM Lechan, PR Larsen** 2001 Regional physiological adaptation of the central nervous system deiodinases to iodine deficiency. Am J Physiol Endocrinol Metab. 281:E54-61

**RP Peeters, EC Friesema, R Docter, PJ Meier, G Hennemann, TJ Visser** 2002 Effects of thyroid state on the expression of hepatic thyroid hormone transporters in rats. Am J Physiol Endocrinol Metab. 283:E1232-8

**D Mesotten, PJ Wouters, RP Peeters, KV Hardman, J Holly, RC Baxter, G Van den Berghe** 2004 Regulation of the somatotropic axis by intensive insulin therapy during protracted critical illness. J Clin Endocrinol Metab. 89:3105-13.

**GG Kuiper, MH Kester, RP Peeters, TJ Visser** 2005 Biochemical mechanisms of thyroid hormone deiodination. Thyroid 15: 787-98

**BC Appelhof, RP Peeters, WM Wiersinga, TJ Visser, EM Wekking, J Huyser, AH Schene, JG Tijssen, WJ Hoogendijk, E Fliers** 2005 Polymorphisms in type 2 deiodinase are not associated with well-being, neurocognitive functioning and preference for combined T4/T3 therapy. J Clin Endocrinol Metab. Sep 6; [Epub ahead of print]

**DC Thijssen-Timmer, RP Peeters, PJ Wouters, F Weekers, TJ Visser, E Fliers, WM Wiersinga, O Bakker, G Van den Berghe** Thyroid hormone receptor alpha splice variants in livers of critically ill patients. *Submitted*

**I Vanhorebeek, RP Peeters, S Vander Perre, I Jans, PJ Wouters, K Skogstrand, TK Hansen, R Bouillon, G Van den Berghe** Cortisol response to critical illness: effect of intensive insulin therapy. *Submitted*

**JP Brouwer, BC Appelhof, RP Peeters, WJ Hoogendijk, J Huyser, AH Schene, JG Tijssen, R Van Dyck, TJ Visser, WM Wiersinga, E Fliers** Thyrotropin, but not a polymorphism in type II deiodinase, predicts response to paroxetine in major depresssion. *Submitted*

**WM van der Deure, AG Uitterlinden, A Hofman, HA Pols, RP Peeters, TJ Visser** Polymorphisms in thyroid hormone pathway genes are associated with bone mineral density and fracture risk: the Rotterdam Study. *Submitted*

**FW Wassen, RP Peeters, R Hume, GG Kuiper, TJ Visser** Type I iodothyronine deiodinase splice variants in human tissues. *Submitted*

## Other publications

**J Broeren en R Peeters** 1999 De goede attitude van de co-assistent. Medisch Contact 36: 1207-10

## Curriculum Vitae

| | |
|---|---|
| 1975 | Geboren te Rotterdam |
| 1987-1993 | Gymnasium Erasmianum, Rotterdam |
| 1993-1998 | Doctoraalfase Geneeskunde, Erasmus Universiteit Rotterdam (1995-1996: praeses Medische Faculteits Vereniging Rotterdam) |
| 04/1997 - 05/1998 | Afstudeeronderzoek naar de regulatie van transport van schildklierhormoon, Afdeling Inwendige Geneeskunde, Erasmus MC, o.l.v. Prof.dr. T.J. Visser |
| 05/1998 - 12/1998 | Onderzoek naar de invloed van type 2 deiodase en schildklierhormoon op het hart, Brigham and Woman's Hospital, Harvard Medical School, Boston, Massachusetts, USA, o.l.v. Prof. P.R. Larsen, MD |
| 2000 | Lid Federatiebestuur van de Koninklijke Nederlandse Maatschappij tot bevordering der Geneeskunst (KNMG), voorzitter KNMG-studentenplatform |
| 10/2000 | Artsexamen Erasmus Universiteit Rotterdam (Cum Laude) |
| 10/2000 – 05/2001 | Arts-assistent Interne, Ikazia Ziekenhuis Rotterdam |
| 05/2001 – 05/ 2003 & 05/2004 – 05/2005 | Promotieonderzoek Afdeling Inwendige Geneeskunde, Erasmus MC Rotterdam, met een persoonsgebonden AGIKO-stipendium van Zon-MW (920-03-146). Onderwerp: Regulatie van de bioactiviteit van schildklierhormoon. Promotor: Prof.dr.ir. T.J. Visser. |
| 5/2003 – 05/2004 & 05/2005 – heden | Opleiding tot internist, Erasmus MC Rotterdam Opleiders: Prof.dr. H.A.P. Pols en Dr. J.C.L.M. van Saase |

*Erkenningen:*

* Endocrine Society Travel Grant Award, San Fransisco, USA, juni 2002
* Young Investigators Travel Grant Award, Los Angeles, USA, oktober 2002
* Young Investigators Award, Istanbul, Turkey, September 2004
* Young Investigators Travel Grant Award, Vancouver, Canada, oktober 2004
* Best Research Abstract Award, 74$^{e}$ Internisten dagen, Maastricht, april 2005