Establishment, validation and application of immunological and LC-MS/MS-based detection methods to study the role of human aromatic L-amino acid decarboxylase as an enzyme potentially involved in thyronamine biosynthesis

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Contents

Zusammenfassung

Thyronamine (TAM) sind eine neue Molekülklasse, die endokrinologische und metabolische Prozesse miteinander vereinen. Der biologisch aktive Metabolit 3-Iod-L-Thyronamin (3-T_1AM) wird durch eine kombinierte Deiodierung und Decarboxylierung von Schilddrüsenhormonen (TH) gebildet. 3-T_1AM ist als 'kühlender' Metabolit in seiner pharmakologischen Wirkungsweise das Gegenstück zum klassischen 'thermogenen' TH 3,3',5-Triiod-L-Thyronin (T_3). Existierende Methoden zum Nachweis und zur Quantifizierung von 3-T_1AM im menschlichen Serum sind immer noch umstritten. Auch die an der Biosynthese vermutlich beteiligte TH-Decarboxylase konnte noch nicht identifiziert werden.

Für die gleichzeitige Identifizierung und Quantifizierung von TH und TAM Profilen in biologischen Proben wurde die Flüssigchromatographie-Tandem-Massenspektrometrie (LC-MS/MS) verwendet. Gleichzeitig wurden mehrere präanalytische Methoden für eine vollständige Extraktion von 3-T_1AM aus humanem Serum getestet. In der bisherigen präanalytischen Aufarbeitung humaner Proben liefern weder Flüssig-Flüssig- noch Festphasenextraktionen reproduzierbare Ergebnisse des 3-T_1AM-Gehalts in humanem Serum. Mit der Entwicklung eines schnellen und spezifischen Extraktionsverfahrens und nachfolgender Detektion mittels LC-MS/MS gelang der gleichzeitige Nachweis der häufigsten TH in einzelnen humanen Serum-Proben, d.h. 3,3', 5,5'-Tetraiod-L-Thyronin (Thyroxin, T_4), T_3, 3,3`,5`-Triiod- L-Thyronin (rT_3) und 3,5-Diiod-L-Thyronin (3,5-T_2). Parallel dazu wurden monoklonale Antikörper (MAb) gegen 3-T_1AM entwickelt und charakterisiert, auf deren Basis ein hoch sensitiver quantitativer 3-T_1AM MAb Chemilumineszenz-Immunoassay (CLIA) entstand. Ergebnisse aus klinischen Kollektiven zeigen, dass 3-T_1AM in menschlichem Serum im nanomolaren Konzentrationsbereich vorkommt und dass 3-T_1AM bei Patienten außerhalb der Schilddrüse produziert wird. Jedoch konnte mittels LC-MS/MS auch endogenes 3-T_1AM in murinen Schilddrüsen nachgewiesen werden. Viele Forscher gehen davon aus, dass die aromatische L-Aminosäure Decarboxylase (AADC) die Synthese von TAM über Decarboxylierung von TH katalysiert. Diese Hypothese wurde durch Inkubation von rekombinanter humaner AADC mit mehreren TH unter vielen verschiedenen experimentellen Bedingungen getestet. In keinem der Experimente konnte funktionell aktive AADC die Decarboxylierung von TH katalysieren, so dass die ursprüngliche Hypothese widerlegt wurde. Diese *In-vitro*-Beobachtungen werden durch die Feststellung unterstützt, dass 3-T_1AM auch in Plasma-Proben von Patienten mit AADC-Mangel detektiert wird.

Zusammenfassend ist die Bestimmung von 3-T_1AM in humanem Serum mittels LC-MS/MS aufgrund der nicht reproduzierbaren präanalytischen Probenaufbereitung problematisch. In dieser Arbeit wird der erste MAb-basierte 3-T_1AM assay vorgestellt, der 3-T_1AM zuverlässig in humanem Serum quantifiziert. AADC, der erste Kandidat für die Decarboxylierung von TH, ist wahrscheinlich nicht an der Biosynthese von TAM beteiligt. TAM werden vermutlich mit Hilfe einer anderen, noch nicht bekannten, Decarboxylase gebildet.

Schlagwörter

Thyronamine

3-Iod-L-thyronamin

Thyroxin

Schilddrüsenhormonmetabolismus

Flüssigchromatographie mit Massenspektrometrie-Kopplung

Monoclonaler Antikörper

Chemilumineszenz-Immunoassay

Aromatische L- Aminosäure Decarboxylase

Abstract

Thyronamines (TAM) are a new class of molecules linking endocrinology and metabolism. Combined deiodination and decarboxylation of thyroid hormones (TH) generates a biologically active 'cooling' metabolite, 3-iodo-L-thyronamine (3-T_1AM), whose pharmacological actions are opposite to those induced by the classical thermogenic TH 3,3`,5-triiodo-L-thyronine (T_3). It remains controversial, which methods are able or not to reliably detect 3-T_1AM in human serum, and the presumed TH decarboxylase is still elusive.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for the simultaneous identification and quantification of TH and TAM profiles in biological samples. Several preanalytical methods were tested for complete extraction of 3-T_1AM in human serum. Thus far, neither liquid-liquid nor solid-phase extraction methods allowed reproducible extraction of 3-T_1AM from human serum samples in the preanalytical sample workup. Nevertheless, a rapid and sensitive extraction procedure was developed for detection of the major TH, 3,3`,5,5`-tetraiodo-L-thyronine (thyroxine, T_4), T_3, 3,3`,5`-triiodo-L-thyronine (rT_3) and 3,5-diiodo-L-thyronine (3,5-T_2), by LC-MS/MS in a single human serum sample. In parallel, monoclonal antibodies (MAb) targeting 3-T_1AM were developed and characterized, and a highly specific quantitative 3-T_1AM MAb-based chemiluminescence immunoassay (CLIA) was developed. Studies in clinical cohorts provide evidence that 3-T_1AM is present in human serum in the nM concentration range and that 3-T_1AM is produced extrathyroidally; however, using LC-MS/MS, endogenous 3-T_1AM was also found in murine thyroid glands. Many researchers have reasoned that the aromatic L-amino acid decarboxylase (AADC) mediates TAM synthesis via decarboxylation of TH. This hypothesis was tested by incubating recombinant human AADC with several TH under extensively varied experimental conditions. In all tested conditions, functionally active AADC failed to catalyze the decarboxylation of TH, thus refuting the initial hypothesis. These *in vitro* observations are supported by the finding that 3-T_1AM is also present in plasma samples of patients with AADC deficiency.

In summary, 3-T_1AM detection in serum using LC-MS/MS encounters preanalytical problems. The first MAb-based 3-T_1AM CLIA is presented, which reliably quantifies 3-T_1AM in human serum. AADC, the first candidate enzyme for TH decarboxylation, is likely not involved in TAM biosynthesis. Hence, TAM are probably formed by another, yet unknown, decarboxylase.

Keywords

Thyronamines

3-iodo-L-thyronamine

Thyroxine

Thyroid hormone metabolism

Liquid chromatography tandem mass spectrometry

Monoclonal antibody

Chemiluminescent immunoassay

Aromatic L-amino acid decarboxylase

1 Introduction

1.1 The thyroid gland as an endocrine organ

The thyroid gland is a richly vascularized organ and the largest endocrine gland in the human body. This butterfly-shaped organ consists of two elongated oval lobes lying on either side of the trachea. These lobes are connected near their poles by a thin isthmus crossing the trachea ventrally (Figure 1A). Macroscopically, the lobes of the thyroid consist of numerous spherical follicles of varying size which represent the structural unit of the thyroid gland (Figure 1B). The follicles consist of a polarized epithelial monolayer of cuboidal thyrocytes and are filled with a homogeneous, slightly acidophilic colloid (Figure 1C). This protein-rich colloid contains a large amount of thyroglobulin (Tg), a 660 kDa glycoprotein rich in tyrosine residues that serve as the scaffold for thyroid hormone (TH) synthesis. The interfollicular space is filled with connective tissue and is highly vascularized. Additionally, the hormone calcitonin is produced in the parafollicular cells (c cells) of the thyroid which migrate into the thyroid gland during development and descend from the neural crest (Figure 1C). Upwards from the isthmus, a pyramidal lobe is also often present. A fibrous or muscular band frequently connects the pyramidal lobe to the hyoid bone.

The most outstanding specialty of the thyroid gland is its ability to concentrate a large amount of iodide for the synthesis of TH. The thyroid gland is the primary source of all iodine-containing compounds or their precursors whereas peripheral tissues are the source of TH metabolites. The synthesis of TH occurs at the apical surface of the follicular cells and iodinated TH-containing Tg is deposited and stored as the colloid. TH are the main endogenous iodine-containing compounds with established physiologic significance in vertebrates.

Figure 1: The position of the thyroid gland and histological structures.

Gross anatomy of a normal thyroid gland (A) and histological appearance of thyroid follicles (B+C). Figure 1A was adopted from (Nussey and Whitehead, 2001).

1.2 Thyroid hormones (TH)

TH, found in all chordate animals, are tyrosine-based hormones produced by the thyroid gland and are critical factors of brain and somatic development in newborns and of metabolic activity in adults. They occur in two forms, the inactive pro-hormone 3,3',5,5'-tetraiodo-L-thyronine (thyroxine, T_4) and the biologically active 3,3',5-triiodo-L-thyronine (T_3), differing in the number of bound iodine atoms.

1.2.1 Biosynthesis of thyroid hormones

TH biosynthesis, storage, and release are regulated via the hypothalamus-pituitary-thyroid (HPT) axis and stimulated by thyroid-stimulating hormone (TSH) and the TSH-releasing-hormone (TRH) (Figure 2 A). The HPT axis allows central control of TH production and secretion by the thyroid gland and thus of the TH concentration in the circulation. In this axis, the hypothalamus produces TRH that stimulates the TSH secretion by the anterior pituitary, which in turn stimulates TH production by TH producing follicles of the thyroid gland. Through a negative feedback loop inhibiting TRH and TSH, TH regulate their own production.

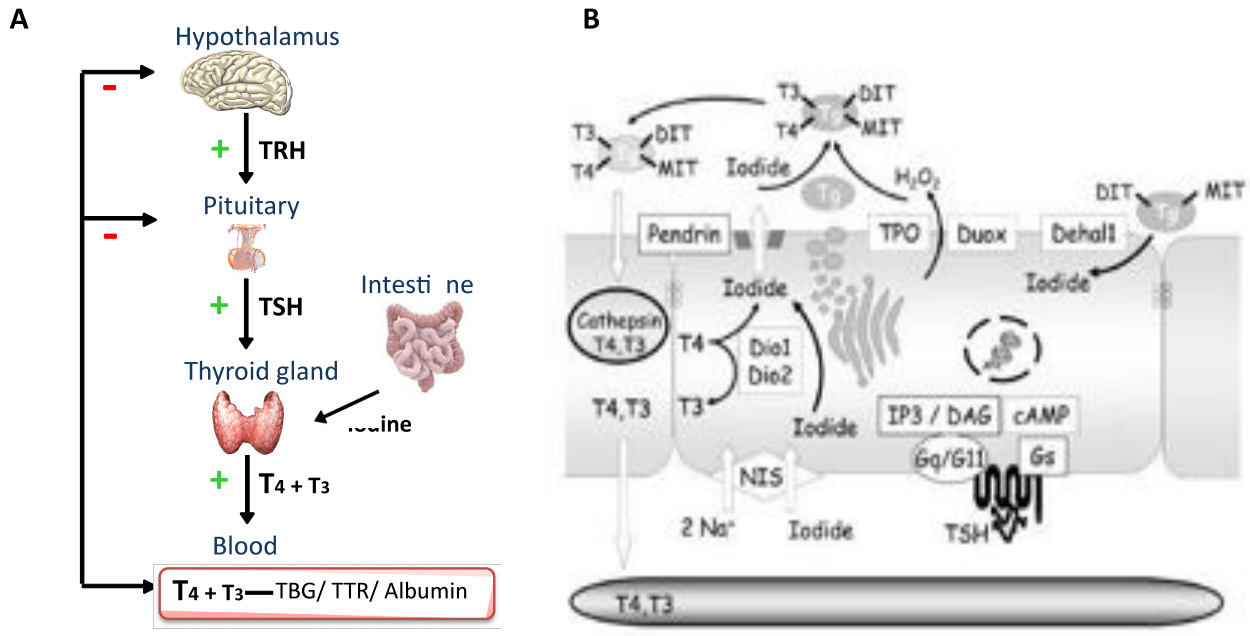


Figure 2: Hypothalamus-pituitary-thyroid axis (A) and thyroid hormone biosynthesis (B).

Dehal: dehalogenase, Dio: deiodinase, DIT: diiodo-tyrosyl-residue Duox: dual oxidase, MIT: monoiodo-tyrosyl residue, NIS: sodium/iodide symporter, Tg: thyroglobulin, TPO: thyroid peroxidase (modified from (Schomburg and Köhrle, 2008)).

The TSH-receptor (TSHR) is localized at the basolateral membrane of thyroidal follicular cells. As a member of the G-protein-coupled receptor family, it is able to trigger both the Gs as well as the Gq/G11 pathway, resulting in stimulation of cAMP/proteinkinase A and phospholipase C (PLC) signaling cascades, respectively. The cAMP pathway activates growth regulation and differentiation as well as TH secretion. The PLC pathway activates H_2O_2 generation and iodination through the PLC-dependent 1,4,5-inositol-tris-phosphate (IP_3) Ca^{2+}/diacylglycerol (DAG) pathway (Ohye and Sugawara, 2010).

A crucial step in TH biosynthesis is the iodination of selected tyrosine residues of Tg. Therefore, two iodide transporters, the sodium/iodide symporter (NIS, SLC5A5) in the basolateral membrane (Dai, et al., 1996; Dohan, et al., 2003) and the anion exchanger pendrin (SLC26A4) in the apical membrane of epithelial thyroctes (Gillam, et al., 2004; Royaux, et al., 2000) convey iodide to the colloid lumen (Figure 2B). NIS co-transports two sodium cations with one iodide anion against an electrochemical sodium gradient as driving force, leading to an exceptionally high iodide accumulation by thyroid follicles. To produce the sodium gradient, the required energy is provided by the ouabain-sensitive Na^+/K^+-ATPase. In the follicular lumen, iodination of Tg tyrosyl residues and covalent coupling of these tyrosyls, are performed by the heme-containing enzyme thyroid peroxidase (TPO). The first step yields monoiodotyrosine (MIT) and diiodotyrosine (DIT). Only selected properly spaced MIT and DIT in Tg participate in the second coupling reaction forming T_4 and T_3 (Taurog, et al., 1996). For this purpose, H_2O_2 is generated at the apical membrane by NADPH-dependent dual oxidases (DUOX 1/2) (De Deken, et al., 2000; Ohye and Sugawara, 2010). Excess MIT and DIT are deiodinated by an iodotyrosine dehalogenase 1 (Dehal1) after their proteolytic liberation from Tg inside the thyrocytes and generated iodide is recycled (Gnidehou, et al., 2004).

1.2.2 Serum transport and cellular uptake of thyroid hormones

After iodination and coupling of tyrosyl residues, the hormone-containing Tg is stored in the colloid lumen until hormone liberation. For TH liberation, hormone-containing Tg or peptides are internalized into epithelial cells and degraded by cathepsins (Brix, et al., 2001; Friedrichs, et al., 2003). Thereby, TH are liberated and can subsequently be released into the blood stream by TH transporters, where they bind to carrier proteins secreted by the liver like albumin, transthyretin (TTR) or thyroxine binding globulin (TBG). Only small amounts of TH

(3% of T_4, 6% of T_3) are bound to lipoproteins (very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL)) (Benvenga, et al., 1988). However, the vast majority of secreted hormone consists of T_4 with a concentration range between 70-150 nM versus 1-3 nM T_3 in human serum. Thus, total serum T_4 concentration is about 60-fold higher than total T_3 (Yen, 2001). These differences are compensated by a reduced affinity of T_3 to TH binding proteins. 0.03% of the circulating T_4 is unbound whereas 0.3% of total plasma T_3 is unbound (Yen, 2001).

The prevailing hypothesis states that free TH concentrations are relevant for TH action and are recognized by their target cells (Ekins, 1992; Mendel, et al., 1988). Opposed to the previous concept that the lipophilic but charged TH enter target cells by passive diffusion, it has been shown that TH require plasma membrane transport proteins. Although several putative TH transporters have been identified, only the monocarboxylate transporter (MCT8) (Friesema, et al., 2003), MCT10 (Friesema, et al., 2008), the L-type amino acid transporters (LAT 1/2) (Friesema, et al., 2001) and the organic anion-transporting polypeptide (OATP) (van der Deure, et al., 2008) displayed high specificity/affinity towards TH transport.

1.2.3 Systemic and local effects of the active thyroid hormone T_3

Once TH has been transported into the cell, TH can bind to nuclear TH receptors (TR) and elicit biological responses. T_3 actions are mediated by two T_3 receptors, TRα and TRβ, which are located on different somatic chromosomes and act as hormone-inducible transcription factors. The different forms of TR are expressed widely in tissue- and developmental stage-specific patterns. These TR bind T_3 with high affinity and mediate TH regulated gene transcription. The TR bind to defined TH responsive elements (TRE) on the DNA, thus changing the expression of target genes. Depending on the structure and sequence of the TRE, ligand dependent or ligand-independent activation or also repression can be observed (Yen, et al., 2006). Most cell types express TR, and thus are TH responsive. The most important target tissues of TH are the central nervous system, white and brown adipose tissue, bone, skeletal muscle and cardiovascular system. In this way, TH influences metabolism, energy and oxygen consumption, bone growth and development. It increases cardiac functions, supports formation and activity of adipose tissue, stimulates liver functions and promotes brain development (Yen, 2001). Amphibian metamorphosis for example is an important example of TH

actions on development (Becker, et al., 1997). In homeothermic species, TH additionally acquired a role in metabolic regulation including thermogenesis (Kim, 2008).

Most of the biological effects of T_3 are mediated by TR regulation of target gene transcription in the nucleus. However, this classical concept of genomic TH action has become more diverse including now also the rapid non-genomic actions of T_3 and T_4 (Davis and Davis, 1996). Non-genomic actions are independent of ligand binding to nuclear TR and are initiated outside the nucleus but may end in cellular actions that are nucleus-mediated. The effects can be classified into TR-dependent or TR-independent pathways. The TR-dependent pathway rely on the cytosolic presence of the classical TR which can functionally interact with other kinase-signaling (mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK1/2), Src kinase, phosphatidyl-inositol 3-kinase (PI3K)) pathways (reviewed in (Davis and Davis, 1996)). The TR-independent effects are mediated by specific membrane receptors for TH unrelated to the classical TR. As receptor for both T_4 and T_3, the plasma membrane receptor on integrin $\alpha v\beta 3$ has been identified to mediate the stimulating effects of TH on cell growth, proliferation, and angiogenesis (Bergh, et al., 2005; Davis, et al., 2006; Davis, et al., 2009).

1.2.4 Metabolism of thyroid hormones

The TH T_4 is synthesized in and secreted from the thyroid gland. It is the main secretory product of the thyroid gland in all vertebrates (Engler and Burger, 1984). In humans it is assumed, that only approximately 20% of circulating T_3 originate directly from the thyroid (Laurberg, 1984), while the thyroidal secretion of the T_3 isomer 3,3',5'-triodo-L-thyronine (reverse T_3, rT_3) is insignificant (Chopra, 1976).

The main metabolic pathway for the peripheral T_4 activation is reductive monodeiodination, whereby a hydrogen atom is exchanged for an iodine atom. Three selenocysteine-containing selenoproteins known as iodothyronine deiodinases (DIO 1, 2, 3) have been identified to exert this function. They show distinct tissue distributions, physiological functions and catalytic specificities (Figure 3) (Bianco and Kim, 2006; Gereben, et al., 2008; Köhrle, 2000; Köhrle, 2002; Köhrle, 2007). However, not only T_4 but also T_3 and rT_3 are substrates of deiodination reactions yielding further interesting TH metabolites, namely diiodo-L-thyronines (3,5-T_2, 3,3`-$T_2$, 3`,5`-$T_2$), monoiodo-L-thyronines (3-$T_1$, 3`-T_1), and L-thyronine (T_0).

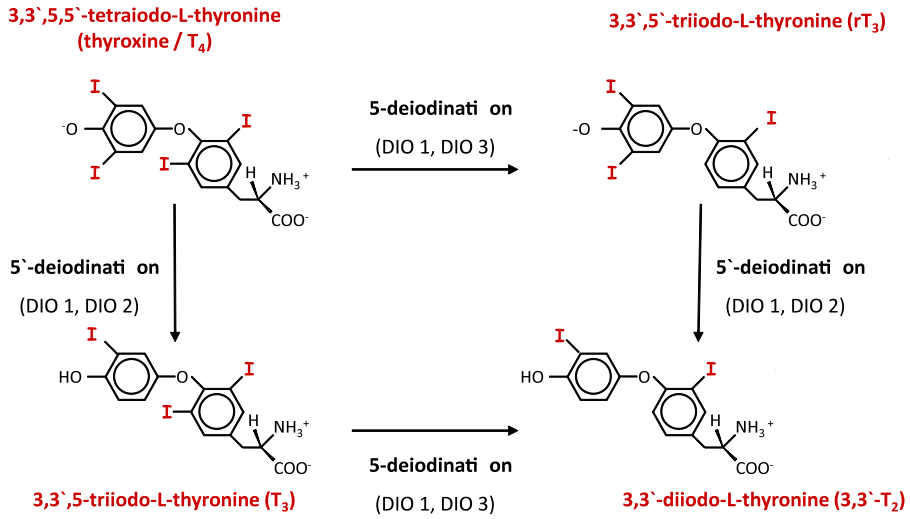


Figure 3: Regulation of T_3 bioavailability by local deiodinase activity.

Deiodinases remove iodide (red) from the phenolic "outer" or tyrosyl "inner" ring of iodothyronines.

However, DIO do not deiodinate the iodinated tyrosines MIT and DIT (Solis, et al., 2004). The deiodination pathway is responsible for degrading >70% of the T_4 secreted by the thyroid gland. The remaining T_4 is metabolized by alternative pathways (Figure 4) (Chopra, et al., 1978). These pathways are based on side chain modifications, their oxidative cleavage at the diphenylether link or conjugation of the 4'-phenolic group with sulfate or glucuronic acid (Visser, 1994; Visser, 1996; Wu, et al., 2005).

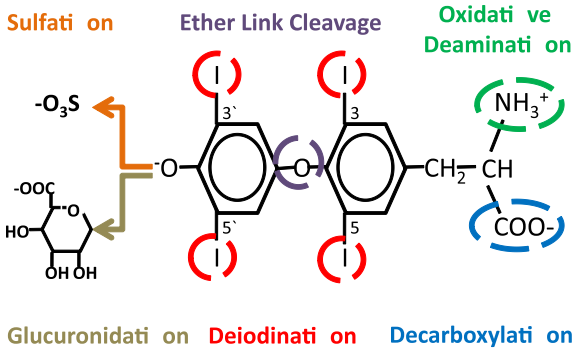


Figure 4: Pathways of thyroid hormone metabolism.

TH metabolites are generated by conjugation, i.e. sulfation or glucuronidation, deiodination, ether link cleavage or side chain modifications, i.e. deamination or decarboxylation. Modified from (Wu, et al., 2005).

Sulfation and glucuronidation are so-called phase II detoxication reactions aimed to increase the water-solubility of the TH to facilitate their biliary and/or urinary clearance (Visser, 1994). The levels of TH sulfate in plasma, bile and urine are normally very low because of the rapid degradation of these conjugates by DIO1. In contrast to the TH sulfates, TH glucuronides are efficiently excreted in the bile. However, after intestinal hydrolysis of the TH glucuronides by bacterial ß-glucuronidases, part of the liberated TH is reabsorbed and again biologically active, constituting an enterohepatic cycle (Visser, 1994; Wu, et al., 2005).

Furthermore, there are two variations of TH side chain modification, concerning either the amine- or carboxyl group. Side chain decarboxylation combined with deiodination of TH leads to the formation of a novel class of biological active metabolites called thyronamines (TAM) (Piehl, et al., 2011; Saba, et al., 2010; Scanlan, et al., 2004). These metabolites influence the physiological manifestations of TH actions by inducing effects opposite from those stimulated by T_3 (Liggett, 2004; Piehl, et al., 2011). It has been shown that TAM are substrates for DIO (Piehl, et al., 2008), sulfotransferases (SULT) (Pietsch, et al., 2007), monoamine oxidases (MAO) and semicarbazide-sensitive amine oxidase (SSAO) (Saba, et al., 2010; Wood, et al., 2009). However, no decarboxylating enzyme (s) catalyzing the conversion of TH to TAM have been identified so far.

Oxidative deamination of TH generates iodoacetic acids (TAc), tetraiodothyroacetic acid (tetrac), triiodothyroacetic acid (triac) and diiodothyroacetic acid (diac) etc., which are habitually present at low levels in human serum (Crossley and Ramsden, 1979; Gavin, et al., 1980; Pittman, et al., 1980; Ramsden and Crossley, 1986). Even though oxidative deamination is described as inactivating pathway for monoamines, triac has significant thyromimetic activity. *In vitro* it is more potent than T_3 for transcriptional regulation by TRβ1 and TRβ2 isoforms, while regulation by TRα1 is equivalent for both ligands (Messier and Langlois, 2000; Moreno, et al., 2008). Interestingly, the cephalochordate Branchiostoma floridae, a marine invertebrate living in warmer coastal areas expresses a TH receptor, which is activated by triac but not by T_3 (Paris, et al., 2008). Moreover, a naturally occurring non-selenodeiodinase catalyzing 5-deiodination of tetrac and triac has been identified. These findings support the hypothesis that triac is a primordial bioactive TH (Klootwijk, et al., 2011).

Ether link cleavage (ELC) is only a minor pathway of TH removal in normal individuals (Balsam, et al., 1983; Kubota, et al., 1985; Wu, et al., 2005). This peroxidase-mediated reaction leads to the formation of DIT from the amino acid site of the molecule (Balsam, et al.,

1983). The liberated iodine is in an oxidized state, allowing it to bind to proteins, resulting in the formation of iodoproteins (Wynn and Gibbs, 1964). Nevertheless, TH breakdown by ECL may support mechanisms for bactericidal activity during severe infections, since in patients with sepsis circulating DIT levels are elevated (Meinhold, et al., 1981; Meinhold, et al., 1988).

1.2.5 Analytics of thyroid hormones

Clinical diagnosis of TH parameters can supply key information on TH status. During the past decades, a lot of diagnostic procedures were presented for experimental and clinical TH analysis. These techniques include the development of simple but sensitive and specific methods for measuring TH. The first method for the quantification of TH was the determination of iodine content in serum. The concentration appeared to be closely correlated with the activity of the thyroid gland (Kydd, et al., 1950). Greater specificity and less interference from non-hormonal iodine-containing compounds were achieved with the development of the butanol-extractable iodine by column technique, which measures the total organic or hormonal iodine (Man, et al., 1954; Man, et al., 1951).

All established chemical and chromatographic methods for serum TH measurement have been replaced by poly-and monoclonal antibody-based immunoassays. The hybridoma technique by Köhler and Milstein offered the opportunity to produce highly specific monoclonal antibodies (MAb). In 1974, they established the hybridoma technique for the production of MAb in cell culture. This method allowed the unlimited production of MAb with predetermined specificity and represents one of the most important inventions in biomedical research during the 1970s. MAb have opened up completely new fields for applied research and allow precise diagnosis of diseases. The principal features of the hybridoma technology are shown in Figure 5.

In 1959, Yalow invented what is still one of the most significant methods of endocrine analysis: the "radioimmunoassay" (RIA). In 1977 she received the Nobel Prize in Physiology or Medicine, together with Guillemin and Schally who identified hypothalamic releasing hormones such as TRH. The first developed immunoassays for TH also used radioisotopic labelings (Brown, et al., 1970; Ekins, 1970; Ekins, et al., 1970).

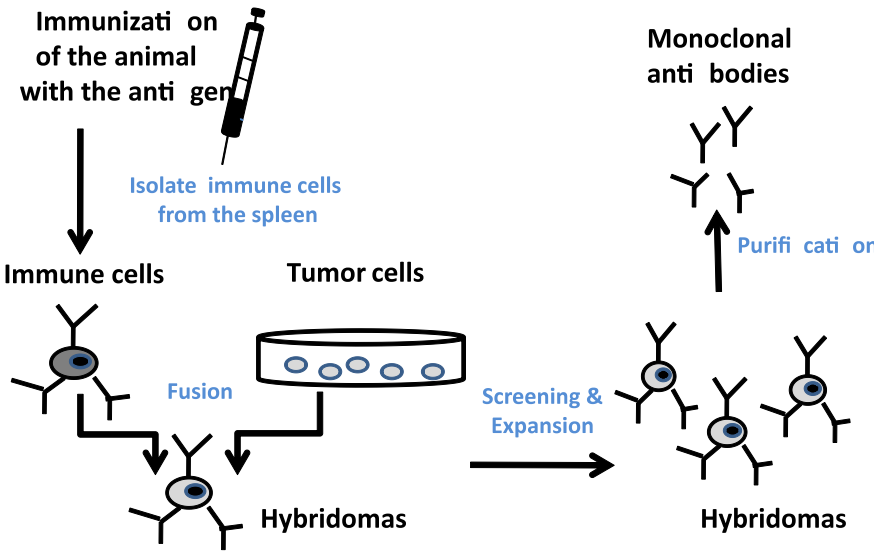


Figure 5: Principal steps in the production of monoclonal antibodies.

Spleen cells are prepared from mice, which have been immunized with a selected antigen. These cells are fused with myeloma cells maintained in culture. The product of this fusion is referred to as a hybridoma. By use of a sensitive detection method the hybridomas which produce the specific antibodies are identified and cloned. A particular hybridoma cell can subsequently be used for unlimited production of a highly specific MAb which can be purified from cell culture supernatant.

Later non-radioisotopic labels, such as enzymes (e.g. alkaline phosphatase, horseradish peroxidase (HRP)) and chemiluminescent molecules (e.g. acridinium esters and luminol) substituted the RIA methods to avoid radioactive exposure and expensive waste management (Hata, et al., 1985; Miyai, et al., 1980; Schall, et al., 1978). However, significant biases for immunoassays have been reported due to either endogenous factors (e.g., abnormal binding proteins, dialyzable protein binding competitors, heterophilic antibodies, autoantibodies) or *in vitro* factors (free fatty acids, bilirubin, immunoglobulins, assay antibodies, analogues, intrinsic dilution) (Yue, et al., 2008). Due to their hydrophobic structure, TH exhibit very strong protein binding. Specific antibodies but also organic solvents used in the preanalytical sample workup along chromatographic or immunological detection procedures disrupt these *in vivo* serum or tissue binding equilibria. Up to now, several independent assays using multiple sample aliquots were necessary to obtain this information for selected TH compounds and metabolites, for which antibodies were available. Sufficiently high discriminative specificity and affinity are needed to detect the most abundant compounds in the samples of interest. The issue of “precise analytical determination” of exact free versus total TH concentrations in body fluids such as blood serum, cerebrospinal fluid, saliva or urine under normal

and pathophysiological conditions fuels an ongoing debate on the rapid and “cost effective estimation” of relevant TH levels for clinical diagnostics.

Therefore, analytical methods using gas chromatography–mass spectrometry (GC–MS) and liquid chromatography tandem mass spectrometry (LC–MS/MS) have been developed to measure TH in human serum or plasma to expand the immunological detection methods (Gu, et al., 2007; Kunisue, et al., 2010; Lembcke, et al., 2005; Soldin and Soldin, 2011; Soldin, et al., 2005; Yue, et al., 2008). LC-MS/MS has emerged as an innovative analytical technology applicable to numerous endocrine analyses (for review see (Krasnoselsky, et al., 2008; Vogeser and Parhofer, 2007; Vogeser and Seger, 2010; Vogeser and Seger, 2012)). This technique identifies the analyte in a sample by the retention time and the mass-to-charge ratio of parent and fragmentation ions (Figure 6). In a typical LC-MS/MS procedure, the sample is taken up by an autosampler and is injected into the stream of mobile phase using one or two pumps (Figure 6). The pumps move the mobile phase and the analyte through a HPLC-column (stationary phase) where the sample compounds are separated chromatographically before they are introduced to the ion source of the mass spectrometer (MS). In the MS instrument, the ion source converts the sample molecules into the gas phase forming charged ions. The ions are separated by electromagnetic fields according to their mass-to-charge ratio. The ions are detected, usually by a quantitative method and the ion signal is processed into mass spectra. In the selected reaction monitoring (SRM) mode, the first analyzer selects only for a specific single parent mass for MS/MS fragmentation and then the second analyzer monitors for a single user defined daughter ion. SRM allows for multiple user defined fragment ions. Quantification of analytes is achieved by determining the ratio of analyte to isotope labeled internal standard and calibration curves.

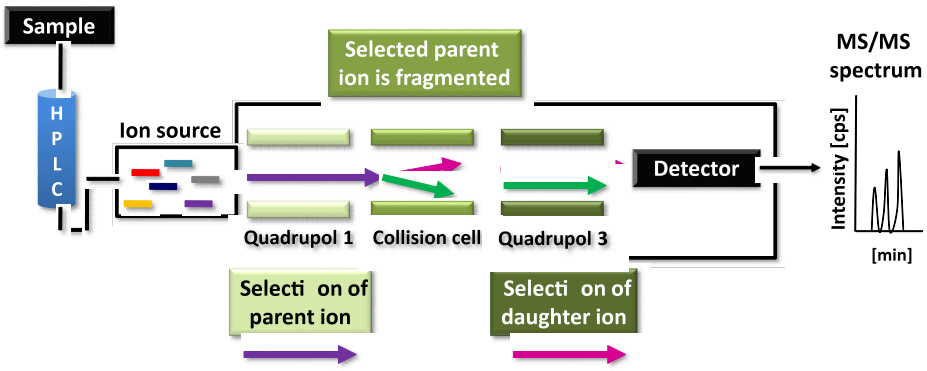
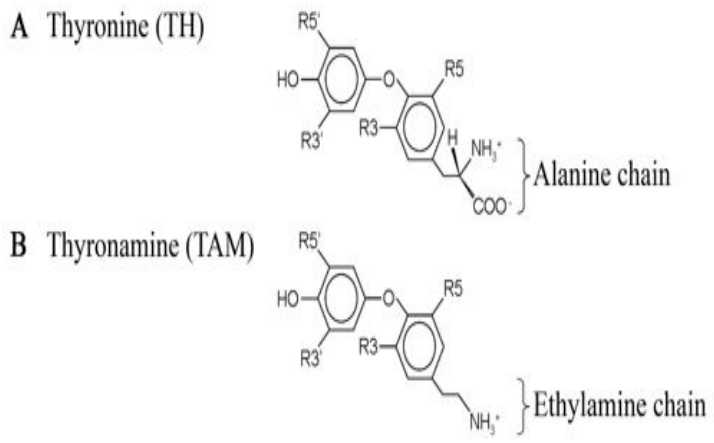


Figure 6: Principle of the Liquid chromatography tandem mass spectrometry (LC-MS/MS).

LC-MS/MS assays are highly accurate and sensitive and they are specific for the analyte. This method has already been shown to be a precise tool for the measurement of serum testosterone, adrenal and gonadal steroids and 25-hydroxyvitamin D concentrations as well as TH (Adamec, et al., 2011; Jonklaas, et al., 2009; Kunisue, et al., 2011; Kunisue, et al., 2010; Piehl, et al., 2008; Surowiec, et al., 2011; Thibeault, et al., 2012; Vogeser and Parhofer, 2007). Nowadays, LC-MS/MS is used in research laboratories as gold standard method in endocrine chemistry. However, clinical routine TH evaluation is still performed by immunoassays. In addition, routine laboratory chemistry methods do not include TH metabolite (e.g. rT_3 and 3,5-T_2) measurements, since the concentration of these TH metabolites are in fact very low in human serum. The introduction of LC-MS/MS based techniques in the analytics of hormones, which circulate in 10^{-15} M (femtomolar) to 10^{-9}M (nanomolar) concentration in various free and bound forms opened discussions and controversies on their 'true concentrations' in body fluids and tissues.

1.3 Thyronamines (TAM)

Thyronamines (TAM) are a novel class of endogenous signaling molecules which can modulate metabolism. TAM were initially synthesized in the 1960-1990 by several groups studying TH metabolism (Cody, et al., 1984; Han, et al., 1987; Meyer and Hesch, 1983; Piehl, et al., 2011; Scanlan, et al., 2004; Tomita and Lardy, 1956). Interest in TAM has significantly increased, since they have been shown to be endogenous molecules in the human body (Scanlan, et al., 2004; Scanlan, 2011). In terms of structure, TAM differ from TH by the absence of the amino acid carboxyl group. Similar to TH, there are nine TAM differing either by the number or by the position of iodine atoms (Figure 7).



Analyte	R3	R5	R3'	R5'
T_0(AM)	H	H	H	H
3-T_1(AM)	I	H	H	H
3'-T_1(AM)	H	H	I	H
3,5-T_2(AM)	I	I	H	H
3,3'-T_2(AM)	I	H	I	H
3',5'-T_2(AM)	H	H	I	I
T_3(AM)	I	I	I	H
rT_3(AM)	I	H	I	I
T_4(AM)	I	I	I	I

Figure 7: Structure and nomenclature of (A) thyroid hormones and (B) thyronamines.
Adopted from Piehl & Hoefig, et al. (Piehl, et al., 2011). R: variable residue, I: iodine, H: hydrogen.

Using LC-MS/MS, two representatives of nine possible TAM isomers, namely 3-Iodo-L-thyronamine (3-T_1AM) and the completely deiodinated product thyronamine L-T_0AM, have recently been detected *in vivo* in serum and tissues of various species such as humans, C57Bl/6 mice, Long-Evans rats, Djungarian hamsters, and guinea pigs (Braulke, et al., 2008; Hoefig, et al., 2011; Saba, et al., 2010; Scanlan, et al., 2004). The endogenous presence of any other TAM *in vivo* has not yet been reported.

1.3.1 Systemic and local effects of thyronamines

Combined deiodination and decarboxylation of TH generates the biologically active 'cooling' metabolite 3-T_1AM. Animal studies showed that a single 3-T_1AM treatment with a pharmacological dose (50 mg/kg body weight) resulted in a rapid and drastic decrease in body temperature (Scanlan, et al., 2004), drop in heart rate (Chiellini, et al., 2007), reduction in cardiac output (Chiellini, et al., 2007; Zucchi, et al., 2008) and an induction of behavioral inactivity in mice (Dhillo, et al., 2009; Scanlan, et al., 2004). These effects were shown to have potentially neuroprotective benefits in case of ischemic injuries such as stroke (Doyle, et al., 2007; Lin, et al., 2011). Furthermore, a decreased respiratory quotient showed a shift from carbohydrate to fat metabolism (Braulke, et al., 2008). Acute alterations in glucoregulatory hormones and glucose metabolism (glucose↑, insulin ↓, glucagon↑) were observed. Decreased TSH and serum TH (T_4 ↓, T_3 ↓) indicated a perturbation of the normal feedback paradigm (Klieverik, et al., 2009; Regard, et al., 2007). Moreover, dose-dependent stimulation of food intake and neuropeptide Y release after intraperitoneal and intracerebroventricular 3-T_1AM administration at lower doses has also been reported (Dhillo, et al., 2009). The pharmacological effects of TAM are summarized in Table 1 and reviewed by Piehl & Hoefig, et al. (Piehl, et al., 2011).

1.3.2 Serum transport and cellular uptake of thyronamines

3-T_1AM appears to prefer different serum proteins for binding as TH (Figure 8). Whereas TH strongly bind to TBG, TTR, albumin and only at minor extent to lipoproteins (Benvenga, et al., 1988), a high fraction of 3-T_1AM (> 90%) is specifically non-covalently bound to apolipoprotein B100 (apoB100) (Figure 8). The dissociation constant (K_D) of 17 nM results in to a low free 3-T_1AM concentration in serum (Roy, et al., 2012). The apolipoprotein occurs in plasma in two main isoforms, apoB100 and apoB48. In humans, apoB100 is expressed in the liver and is present on VLDL and LDL whereas apoB48 is expressed in the intestine and is present on chylomicrons and their remnants (Olofsson and Boren, 2005).

The human circulating apoB100 concentration is 1.5 - 3.0 µM (77 - 153 mg/dL), depending on the lipid inventory which is regulated by nutrition, hormonal influences (estrogens, insulin, growth hormone), metabolic disorders (diabetes and obesity) and genetic background (e.g., cholesteryl ester storage disease, cholesterol ester transfer protein (CETP) deficiency, hypo-

Table 1: Summary of the pharmacological effects of thyronamines.

	Experimental model	Mechanism and/or interpretation	Reference
Central nervous effects			
Hypothermia	C57Bl/6 mice, Djungarian hamsters	Due to a decrease in metabolic rate	(Braulke, et al., 2008; Doyle, et al., 2007; Scanlan, et al., 2004)
Inhibition of monoamine transporters	Transfected cell lines	Hypothesized action as a relevant neuromodulator	(Snead, et al., 2007)
Cardiac effects			
Negative chronotropy*, Negative inotropy,	C57Bl/6 mice, isolated rat hearts	Due to effects on intracellular calcium homeostasis	(Chiellini, et al., 2007; Ghelardoni, et al., 2009; Scanlan, et al., 2004; Zucchi, et al., 2008)
Effects on plasma hormone concentrations			
Hyperglycemia, Hypoinsulinemia, Hyperglucagonemia	Primary human and murine pancreatic β-cells, male Wistar rats, C57Bl/6 and ROSA26PTX mice	Hypothesized central action increasing the sympathetic tone on pancreatic islets	(Klieverik, et al., 2009; Regard, et al., 2007)
Hypothalamus-pituitary-thyroid-axis: TSH ↓, T_4 ↓, T_3 ↓	Male Wistar rats, C57Bl/6 mice	Causing a state reminiscent of non-thyroidal illness	(Klieverik, et al., 2009; Manni, et al., 2012)
Metabolic effects			
Metabolic rate ↓, RQ ↓, Lipid utilization ↑, Fat mass ↓, Ketonuria, mitochondrial O(2) consumption↓	C57Bl/6 mice, Djungarian hamsters Rat liver mitochondria	Hypothesized action as a physiologically relevant modulator of metabolism: change in metabolic fuel utilisation from carbohydrates to mainly lipids	(Braulke, et al., 2008; Venditti, et al., 2011)

Legend: * Conflicting data have been reported for the cardiac effects of T_0AM (Boissier, et al., 1973; Chiellini, et al., 2007; Cote, et al., 1974), ↓ reduction; ↑ increase.

betalipoproteinemia, heterozygous familial hypercholesterolemia, familial combined hyperlipidemia, familial defective apoB100) (Beghin, et al., 2000). ApoB100 serves as a ligand for the LDL receptor (LDLR), which is mainly responsible for the removal of LDL from plasma. Hence, the physiological role of the strong binding of 3-T_1AM to apoB100 may be to provide a mechanism for transportation and entry of 3-T_1AM into target cells via LDLR mediated endocytosis (Roy, et al., 2012). The receptor mediated endocytosis involving apoB100 is a well-established cellular uptake mechanism for small molecule lipids such as cholesterol and triglycerides (Benvenga, et al., 1988).

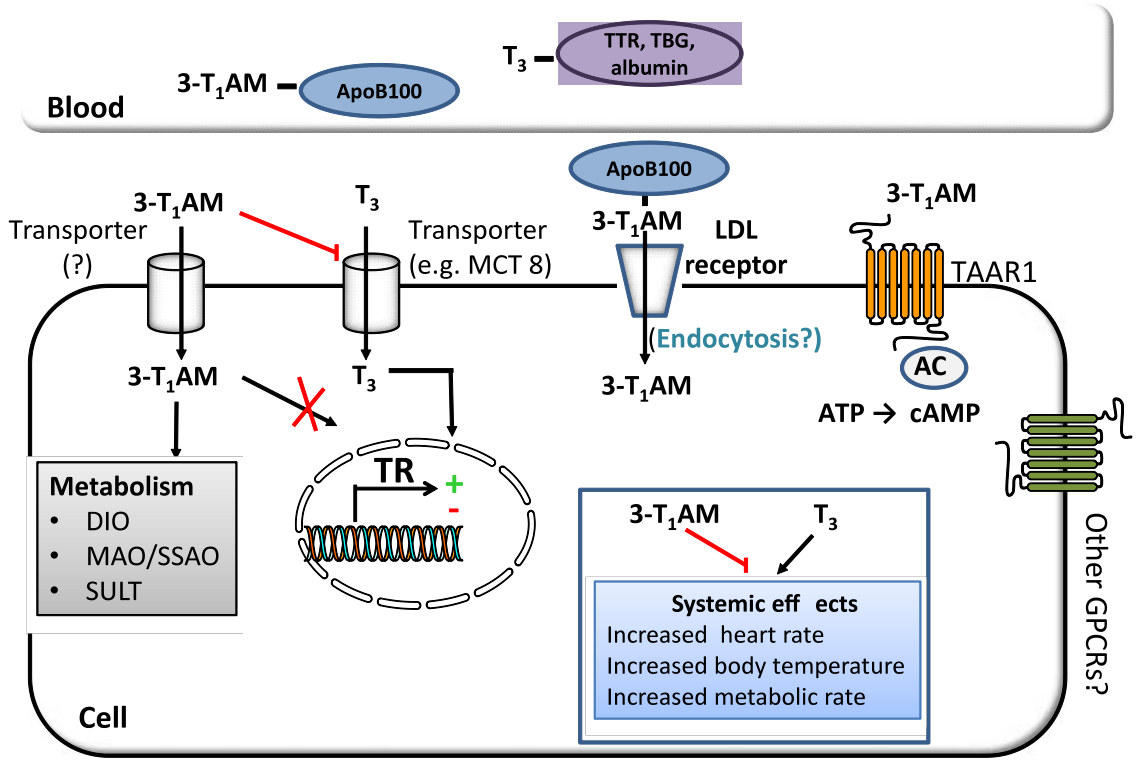


Figure 8: Concept of thyroid hormone and thyronamine action within the cell.

Thyroid hormones are bound in plasma to serum proteins like TBG, TTR and albumin. They enter a target cell via specific transporters, e.g. T_3 uses the monocarboxylate transporter MCT8. Cytosolic T_3 exerts genomic effects via the nuclear TH receptor (TR). TH derivatives like 3-T_1AM modulate the action of T_3, e.g. counter-acting its systemic effects. 3-T_1AM is bound to apolipoprotein B100 (apoB100) and activates the G-protein coupled receptors (GPCRs) of the trace amine associated receptor 1 (TAAR 1) family followed by adenylylcyclase (AC) activation with subsequent rise of cAMP. Moreover, 3-T_1AM is not transported into the cell via the TH transporters but maybe via yet unknown transporters (Ianculescu, et al., 2010; Ianculescu, et al., 2009). 3-T_1AM itself inhibits TH transport and does not bind to the TH receptor. In the cell, 3-T_1AM can be metabolized via deiodination (DIO), oxidative deamination (MAO, SSAO) or conjugation with sulfate groups.

Systemic administration of 3-T_1AM permits its passage through the blood–brain barrier, but it is unknown by which precise mechanism this occurs. Remarkably, 3-T_1AM is not a substrate of TH transporters like MCT8 or MCT10 (Ianculescu, et al., 2010; Ianculescu, et al., 2009; Kinne, et al., 2010; Scanlan, et al., 2004). To identify putative 3-T_1AM transporter(s), a systematic large scale screening analysis of the solute carrier (SLC) transporter family was performed. No single specific TAM transporter was identified from this screen, however, sodium and chloride independent, pH dependent, TAM specific intracellular uptake, may involve multiple transporters (Figure 8) (Ianculescu, et al., 2010; Ianculescu, et al., 2009).

1.3.3 Receptors of thyronamines

The cellular transport mechanism could serve as a signal to terminate 3-T_1AM action at its membrane receptors or provide a way of 3-T_1AM recycling (Ianculescu, et al., 2009). Inside the cell, 3-T_1AM may have other important but still undiscovered roles. The rapid onset of TAM effects led to their classification as non-genomic effects. The structural similarities of TAM with TH and biogenic amines suggested that TAM might signal either via nuclear TH receptors and/or cell membrane receptors of biogenic amines (Snead, et al., 2007). However, 3-T_1AM cannot activate the classical slow acting TH receptors (Scanlan, et al., 2004). No other nuclear receptor activated by 3-T_1AM has been identified up to now. So far, only trace-amine-associated receptor 1 (TAAR1) is activated by 3-T_1AM and signals via adenylylcyclase (AC) activation. However, a recent report rejects the hypothesis that TAAR1 mediates the thermoregulatory response to 3-T_1AM (Panas, et al., 2010). Surprisingly, the previously demonstrated hypothermic cryogenic response to 3-T_1AM administration was maintained in TAAR1 knockout mice to a similar extent and with the same dose response at 25 (~70 µmol) and 50 (~140 µmol) mg /kg body weight as in wildtype mice.

Whether other members of the TAAR family or other plasma membrane receptors mediate this cryogenic 3-T_1AM response, the therapeutic relevance for treatment of stroke, cardiovascular complications and intensive care medicine remains to be studied (Scanlan, 2011). A second receptor proposed to mediate the effects of 3-T_1AM, was the α_{2A} adrenergic receptor ($Adra_{2A}$) which is an established Gαi PCR expressed in many cell types including pancreatic β-cells and the heart (Regard, et al., 2007).

T_3 increases heart rate, basal metabolic rate, and body temperature (Biggins and Koh, 2007; Flamant, et al., 2007). Hence, the effects of TAM are opposite to those of T_3. This could be an indication that T_3 and TAM act to maintain a homeostatic balance, with 3-T_1AM serving as a quick brake to the more gradual stimulatory effects seen with T_3 (Weatherman, 2007). TAM might fine-tune or even antagonize TH effects (Dratman, 1974; Liggett, 2004; Piehl, et al., 2011; Scanlan, et al., 2004). These recent observations significantly expand the potential molecular and metabolic repertoire of TAM. Therefore, the biosynthesis, metabolism and action of 3-T_1AM could be relevant factors in physiological and pathophysiological states like the low-T_3-syndrome (Stathatos and Wartofsky, 2003), severe general and neurological diseases or adaption to food and carbohydrate restriction. Extensively described variations of the T_3 and rT_3 serum levels cannot yet adequately explain the observed metabolic changes in relation to serum TH (Hesch, 1981; Kaptein, et al., 2009; Wartofsky and Burman, 1982).

1.3.4 Biosynthesis of thyronamines

TAM differ from TH only by a carboxylate group. Biosynthesis of TAM from TH therefore appears likely (Figure 9). If TAM were derivatives of TH, decarboxylation of the alanine side chain would be required for their biosynthesis. If the putative decarboxylating enzyme was converting only TH with higher iodine content, deiodination would be directly required to complete 3-T_1AM and T_0AM biosynthesis by removing at least one to four iodine atoms. However, the pathway of TAM biosynthesis is still elusive and controversially discussed (Ackermans, et al., 2010; Hoefig, et al., 2012; Saba, et al., 2010).

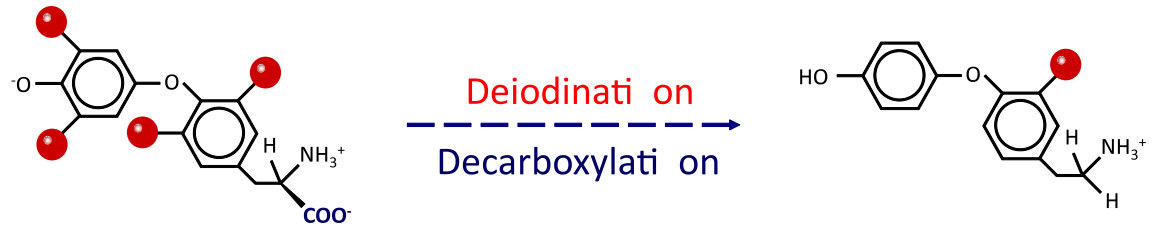


Figure 9: Hypothetical biosynthesis of 3-T_1AM from T_4.

De novo biosynthesis would require partial oxidative iodination and ether-bond coupling of two tyrosyl rings resembling the biosynthesis of TH which occurs bound to their precursor protein Tg (Dunn and Dunn, 2001). Alternatively, *de novo* biosynthesis of 3-T_1AM from T_0AM would require iodination of T_0AM. So far, both reactions have been described only within the thyroid gland, where they occur in the specialized compartment of the thyroidal follicle and involve TPO and DUOX (Ris-Stalpers, 2006). However, neither significant release of TH with lower iodination grade than T_4 or T_3 nor direct secretion of TAM from the thyroid gland has been reported, supporting the hypothesis of extrathyroidal TAM biosynthesis from TH. Further support for this proposal comes from the *ex vivo* experiments where the exposure of H9c2 rat cardiomyoblasts to T_3 but not to T_4 led to very low but detectable 3-T_1AM production (Saba, et al., 2010). Similarly, no 3-T_1AM production could be observed in cultured primary rat thyrocytes and rat FTRL-5 thyrocytes offering T_4 as substrate (Agretti, et al., 2011).

A tentative biosynthetic pathway requires decarboxylation and several deiodination steps to convert e.g. T_4 into 3-T_1AM (Figure 10). Piehl, et al. have previously shown that the DIO are capable of reductively eliminating iodine from all positions in TAM (Piehl, et al., 2008). Normally, these three isozymes convert T_4 into the active T_3 (DIO1 and DIO2 (Köhrle, 2002; Watanabe, et al., 2006)) or into the inactive rT_3 (DIO1 and DIO3). However, the TH decarboxylase, the sequence of reactions and locations of biosynthesis is still elusive at the moment (Wu, et al., 2005).

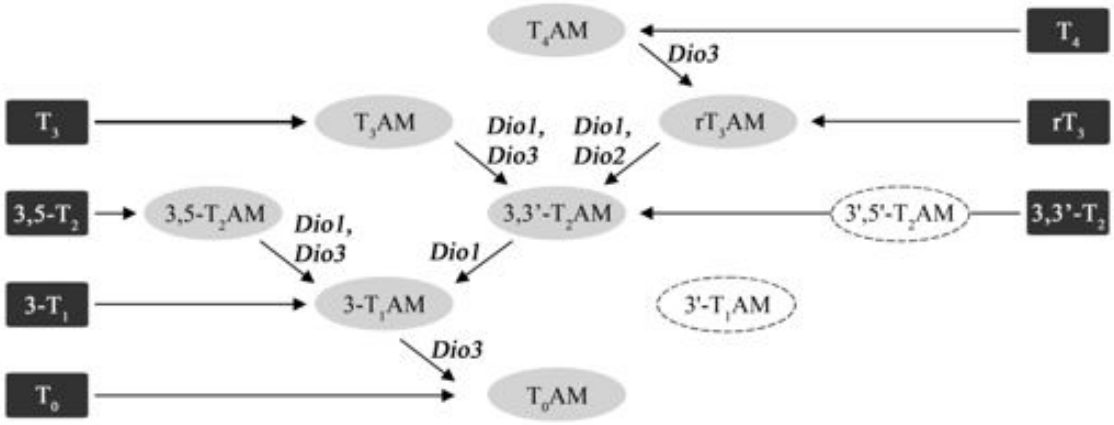


Figure 10: Pathways suggested for thyronamine biosynthesis.

Adopted from Piehl & Hoefig, et al. (Piehl, et al., 2011). Diagonal arrows: phenolic ring deiodination reactions catalyzed by DIO1 or DIO2 and tyrosyl ring deiodination reactions catalyzed by DIO1 or DIO3. Horizontal arrows: putative decarboxylation reactions which might represent the first step of TAM biosynthesis. Dashed lines: TAM which have been excluded as precursors of the biosynthesis of 3-T_1AM and T_0AM.

Decarboxylation is a classical biochemical reaction in human physiology (Table 2). The list of enzymes of the Nomenclature committee of the International Union of Biochemistry and Molecular Biology (IUBMB) registered under EC 4.1.1 (carboxy-lyases) more than 90 known decarboxylase enzymes. For instance, the link between glycolysis and the citric acid cycle is the oxidative decarboxylation of pyruvate to form acetyl CoA. Moreover, amino acids are decarboxylated to primary amines also called biogenic amines. Several low-molecular-weight hormones are synthesized from amino acids. For instance, tyrosine is the precursor for the biosynthesis of dopamine, epinephrine, norepinephrine and as mentioned above TH. Many researchers have therefore reasoned that the aromatic L-amino acid decarboxylase mediates TAM synthesis via decarboxylation of TH (Braulke, et al., 2008; Dratman, 1974; Klieverik, et al., 2009; Scanlan, 2009; Scanlan, et al., 2004).

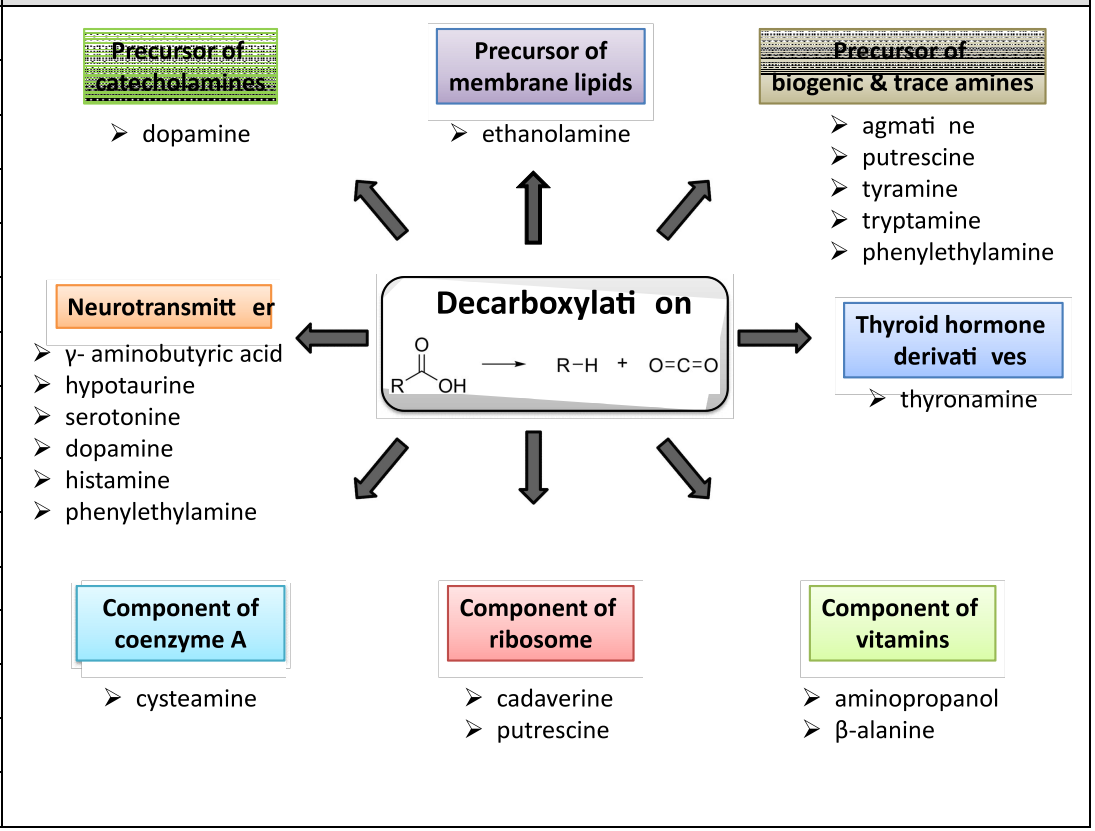
1.3.4.1 Aromatic L-amino acid Decarboxylase

Aromatic L-amino acid decarboxylase (AADC, E.C. 4.1.1.28), also known as L-DOPA decarboxylase (DDC), is a ubiquitous enzyme and requires pyridoxal-5-phosphate (PLP, activated vitamin B6) as cofactor during the conversion of L-DOPA to dopamine or of 5-hydroxytryptophan (5-HTP) to serotonin (Zhu and Juorio, 1995). However, the optimal conditions of pH, temperature and substrate concentration are different for each substrate (Zhu and Juorio, 1995). In addition, AADC is also considered to be involved in the biosynthesis of trace amines such as tryptamine, tyramine and phenylethylamine (Dyck, et al., 1983; Saavedra, 1974). Since 1974, AADC has been proposed as a candidate for TH decarboxylation (Braulke, et al., 2008; Doyle, et al., 2007; Dratman, 1974; Klieverik, et al., 2009; Pietsch, et al., 2007; Scanlan, 2009; Scanlan, et al., 2004). This hypothesis is supported by the broad substrate specificity of AADC and the structural similarity of TAM to biogenic amines, also called monoamines (Figure 11) (Liggett, 2004).

Other similarities between TAM and biogenic amines are: 1) 3-T_1AM activates mouse TAAR1 stably expressed in HEK293 cells, like other biogenic amines (Scanlan, et al., 2004). 2) SULT catalyze the sulfation of many endogenous compounds that include monoamine neurotransmitters, such as dopamine, TH and 3-T_1AM (Pietsch, et al., 2007). 3) 3-T_1AM significantly inhibited dopamine, norepinephrine, and serotonin transport indicating that these substances share the same transport systems (Snead, et al., 2007). 4) MAO and SSAO are promiscuous enzymes that prefer primary amine substrates, and can catalyze the

Table 2: Amino acids, their decarboxylated derivatives and biological function.

Amino acid	Biogenic amine	Enzyme	Biological function
L-phenylalanine	phenylethylamine	AADC	
L-tyrosine	tyramine	tyrosine DC	
L-tryptophane	tryptamine	AADC	
L-histidine	histamine	histidine DC	
L-DOPA	dopamine	AADC	
5-hydroxytryptophane	serotonine	AADC	
arginine	agmatine	arginine DC	
glutamate	γ- aminobutyric acid (GABA)	L-glutamatic acid DC	
ornithine	putrescine	ornithine DC	
cysteinesulfinate	hypotaurine	CSAD	
serine	ethanolamine	serine DC	
threonine	aminopropanol	threonine DC	
asparate	β-alanine	aspartate DC	
cysteine	cysteamine	PDC	
lysine	cadaverine	lysine DC	



Legend: AADC: Aromatic L-amino acid decarboxylase; CSAD: cysteine sulfinic acid decarboxylase; DC: decarboxylase; PDC: Phosphopantothenoylcystein-decarboxylase

oxidation of phenylethylamines and 3-T_1AM (Wood, et al., 2009). 5) Cardiac 3-T_1AM content averaged 6.6 pmol/g wet weight (Saba, et al., 2010), which is about two orders of magnitude lower than myocardial norepinephrine or acetylcholine content, but similar to epinephrine, dopamine, or adenosine content (Chiellini, et al., 2007).

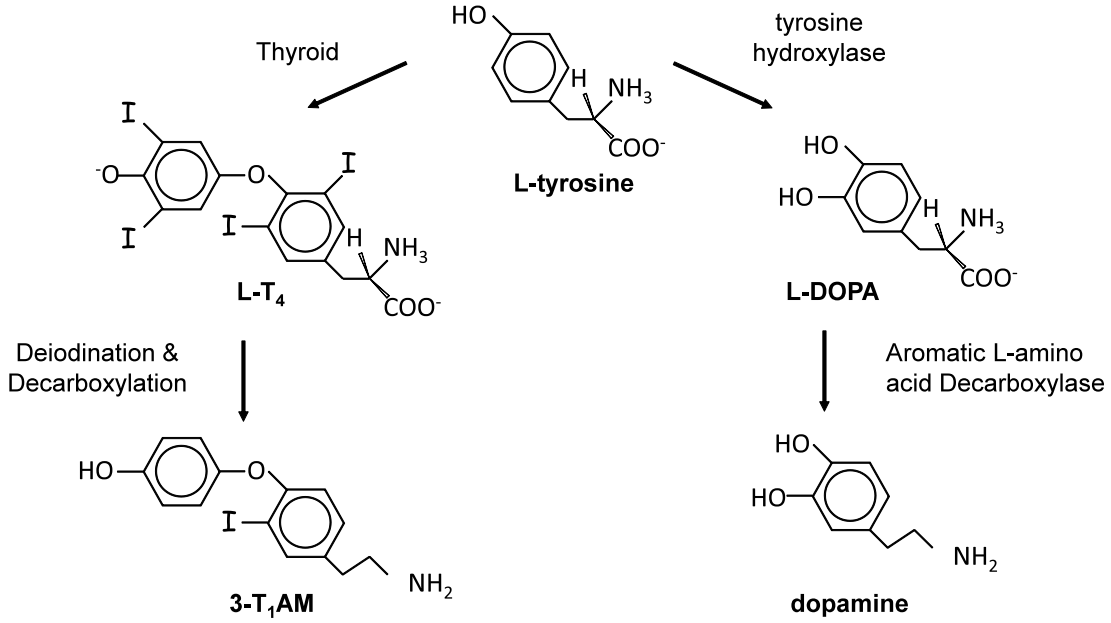


Figure 11: Pathway of dopamine and theoretical thyronamine biosynthesis.

Aromatic L-amino acid Decarboxylase (AADC) catalyzes the conversion of L-DOPA to dopamine (right). 3-T_1AM is structurally related to iodothyronines such as T_4 (left).

AADC deficiency (OMIM #608643) is a rare congenital error of neurotransmitter metabolism and one of the infantile movement disorders. In 1990, Hyland and Clayton reported the first patients with AADC deficiency (Hyland and Clayton, 1990; Hyland, et al., 1992). To date, less than 100 cases have been reported worldwide (Brun, et al., 2010; Korenke, et al., 1997; Lee, et al., 2009; Pons, et al., 2004; Swoboda, et al., 2003; Tay, et al., 2007) with a relatively high occurrence rate in Taiwan (Lee, et al., 2009). These patients are characterized by severe neurometabolic disorders with developmental delay, prominent motor abnormalities, oculogyric crises and autonomic dysfunctions. In addition, they can develop endocrine symptoms, e.g. elevated prolactin, hypoglycaemia and growth hormone deficiency (Ide, et al., 2009; Swoboda, et al., 1999; Swoboda, et al., 2003). These symptoms are caused by defects in the *ADDC* gene (located in 7p12.1p12.3).

The diagnosis of AADC deficiency is made by analysis of neurotransmitters of cerebrospinal fluid (Brun, et al., 2010), the determination of AADC enzyme activity in plasma using both of its substrates, 5-HTP and L-DOPA (Hyland, et al., 1992; Verbeek, et al., 2007) and *AADC* gene mutation analysis (Brun, et al., 2010; Hyland, et al., 1992; Lee, et al., 2009). Nowadays, the preimplantation and prenatal genetic diagnosis of AADC deficiency with an amplification refractory mutation system-quantitative polymerase chain reaction is published to prevent births of AADC deficient patients (Kuo, et al., 2011). Available treatment options are limited (e.g. dopamine agonists, pyridoxine (vitamin B6) and MAO inhibitors) and have only marginal therapeutic effects while the prognosis is uncertain (Brun, et al., 2010; Pons, et al., 2004).

In contrast, AADC becomes the rate-limiting step of dopamine synthesis in patients with Parkinson's disease treated with L-DOPA. Dopamine supplementation therapy was established around 1970 and is still the gold standard for the pharmacotherapy of Parkinson's disease, an age-related movement disorder characterized by decreased levels of the neurotransmitter dopamine (Christine, et al., 2009). AADC activity is inhibited by carbidopa outside the blood brain barrier to inhibit the premature conversion of L-DOPA to dopamine (Nagatsua and Sawadab, 2009). After initial improvement with L-DOPA, many patients with Parkinson disease require higher doses for benefitial effects (Christine, et al., 2009) due to a severe loss of nigrostriatal nerve terminals leading to profound decreased activities of AADC. Hence, many researchers work on a *AADC* gene therapy using adeno-associated virus vector–mediated gene delivery of AADC to restore striatum-selective dopamine production (Christine, et al., 2009; Fan, et al., 2001; Li, et al., 2006; Muramatsu, et al., 2010; Ozawa, 2007).

1.3.5 Analytics of thyronamines

3-T_1AM has already been confirmed as an endogenous compound and was extracted from tissues and blood of several species like humans, mice, and rats (Hoefig, et al., 2011; Saba, et al., 2010; Scanlan, et al., 2004; Soldin, 2009). Using LC-MS/MS, the reported levels of 3-T_1AM and T_0AM in serum ranged from 10^{-12} M (picomolar) to 10^{-9} M (nanomolar) due to different preanalytical extraction methods and device-dependent limits of detection (Table 3).

Table 3: Published methods for the quantification of thyronamines in serum.

Publication	Method	Species	3-T_1AM	T_0AM
(Scanlan, et al., 2004)	Offline SPE + LC-MS/MS	mouse	n.q.	n.q.
(Braulke, et al., 2008)	Offline SPE + LC-MS/MS	hamster	~ 6 nM	n.a.
(Geraci, 2008)	Offline SPE + LC-MS/MS	human	~ 60 nM	n.a.
(Soldin, 2009)	Online SPE + LC-MS/MS	human	~ 0-92.6 pM	n.a.
(Saba, et al., 2010)	Offline SPE + LC-MS/MS	rat	~ 0.3 nM	~0.04 pM
(Ackermans, et al., 2010)	Online SPE + LC-MS/MS	rat/human	n.d.	n.d.
(Galli, et al., 2012)	Offline SPE + LC-MS/MS	human	~ 0.2 nM	n.a.
(Manni, et al., 2012)	Offline SPE + LC-MS/MS	human	~ 0.2 nM	n.a.

Legend: SPE: solid phase extraction; n.a.: not analyzed; n.q.: not quantified, n.d.: not detected

In 2008, Geraci, et al. reported at the American Thyroid Association (ATA) meeting the first endogenous 3-T_1AM concentration in human tissues (higher concentrations in thyroid, skeletal muscle, adipose tissue and prostate ~60 nM, n=2-5) and serum (~60 nM, n=16) (Geraci, 2008) using a solid phase extraction (SPE) and LC-MS/MS method described by DeBarber, et al. 2008 (Figure 12) (DeBarber, et al., 2008).

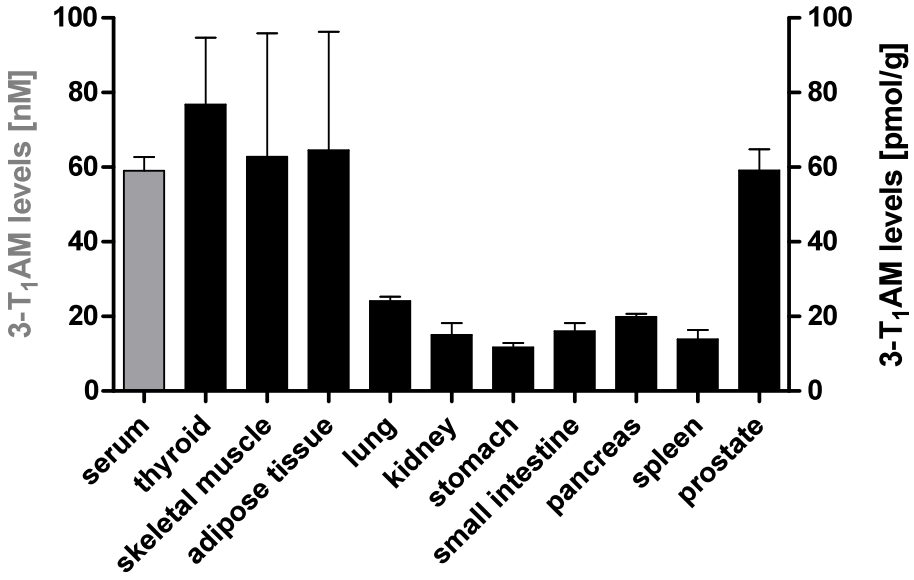


Figure 12: Histogram of 3-T_1AM levels in human serum and tissues.

Modified from (Geraci, 2008). The concentration of 3-T_1AM in human serum is about 60 nM [the grey bar]. Also high tissue concentrations in pmol/g were found in normal human thyroid tissues, suggesting 3-T_1AM may be synthesized by the thyroid gland, and in tissues previously shown to be affected by exogenous 3-T_1AM administration or accumulated such as skeletal muscle and adipose tissues.

In 2009, Soldin, et al. described again at the ATA meeting a novel isotope dilution LC-MS/MS method for the simultaneous identification and quantification of 3-T_1AM, T_4, T_3 and 3,3'-T_2 in biological samples using a highly sensitive triple quadrupole mass spectrometer (API 5000™ LC/MS/MS System) (Soldin, 2009). However, human 3-T_1AM concentrations measured with this method have not yet been published in a peer reviewed article.

The presence of 3-T_1AM in human and animal blood has been challenged by the publication of Ackermans, et al. (Ackermans, et al., 2010). Their online SPE extraction method combined with LC-MS/MS did not detect any endogenous 3-T_1AM or T_0AM in plasma from rats, nor in human plasma or thyroid tissue. They could only find 3-T_1AM and T_0AM in plasma and liver from rats treated with synthetic 3-T_1AM and T_0AM (Ackermans, et al., 2010). These negative findings raised doubts on the biosynthesis pathway and endogenous existence of 3-T_1AM.

Afterwards, 3-T_1AM has again been confirmed as an endogenous molecule in rat blood and tissue (Saba, et al., 2010). The relationship between human 3-T_1AM concentration, TH concentration and some common clinical chemistry variables was investigated in a recent study by Manni, et al. They measured an averaged 3-T_1AM concentration of 0.219 ± 0.012 pmol/ml in healthy patients (n=22). The 3-T_1AM concentration was significantly correlated to tT_4, tT_3, glycated hemoglobin, brain natriuretic peptide and γ-glutamyl transpeptidase (Manni, et al., 2012).

Moreover, in a small cohort of patients, the group showed that 3-T_1AM was significantly higher in diabetic (n=7) vs. non-diabetic patients (n=18) and significantly correlated with HbA1c levels suggesting a potential role of 3-T_1AM in insulin resistance (Manni, et al., 2012). They did not find any significant difference in patients with cardiac dysfunction (Manni, et al., 2012).

The procedure of extracting 3-T_1AM from human samples is analytically complex and time-consuming. The sample capacity is limited and the access to a sensitive mass spectrometer is required. Up to the beginning of this thesis, no immunoassay as a classical TH method to measure 3-T_1AM has been developed and published.

1.4 Aims of the study

TAM have recently been described as endogenous signaling molecules which exhibit great structural similarity to TH, and biosynthesis from TH appears likely. While classically TH are determined via immunological methods, several LC-MS/MS methods have been reported for the detection of 3-T_1AM in serum and tissues of various species. Nevertheless, controversies still exist about the appropriate method of detection as well as serum and tissue levels of endogenously present and exogenously administered 3-T_1AM in body fluids and tissues. A tentative biosynthetic pathway of 3-T_1AM formation requires decarboxylation and several deiodination steps to convert e.g. the pro-hormone T_4 into the most potent TAM, 3-T_1AM.

The three central hypotheses of this project are:

1) TAM are produced from TH by regulated decarboxylation and deiodination.

2) Both classical immunoassay-based methods and novel LC-MS/MS analytics are suitable for direct quantification of TAM in body liquids and biological specimen or after preanalytical sample workup.

3) The decarboxylation of TH to TAM is mediated by the non-selective enzyme AADC.

Consequently the two main scientific goals for this work are:

1) To establish the analytics to measure TAM and TH in different matrix compositions.

2) To provide new insights into the biosynthesis and metabolism of TAM.

As TH and their metabolites like TAM are powerful regulators of growth, development, metabolic function, energy and structural metabolism of the body, the purpose of this project will be to gain more insight into the physiological role and biosynthesis of TAM.

2 Material and methods

Suppliers of the chemicals and reagents (Table 4), TH related compounds (Table 5), laboratory equipment (Table 6), software (Table 7), and buffers (Table 8) used in this project can be found in Tables 4-8.

Table 4: The chemicals and reagents used in this study were of highest purity available and obtained from the following companies

Supplier
Carl Roth (Karlsruhe, Germany)
Sigma-Aldrich (Taufkirchen, Germany)
Merck KGaA (Darmstadt, Germany)
VWR International (Hannover, Germany)
Boehringer (Mannheim, Germany)
Bio-Rad (Munich, Germany)
Fluka (Steinheim, Germany)
Roche Diagnostics GmbH (Penzberg, Germany)
Diagnostic Systems Laboratories (Webster, TX, USA)
Invitrogen GmbH (Karlsruhe, Germany)
PerkinElmer (Waltham, USA)
Serva Feinbiochemika (Heidelberg, Germany)

Table 5: List of TH related compounds used in this study.

Supplier and compound
formula (Berlin, Germany)
T_0, 3-T_1, 3'-T_1, 3,3'-T_2, 3,5-T_2, 3',5'-T_2, T_3, T_3AM, rT_3, rT_3AM, T_4, T_4AM, Tetrac, Triac, Diac
Thomas S. Scanlan, OHSU (Portland, USA)
T_0AM, 3-T_1AM, d_4-3-T_1AM, 3'-T_1AM, 3,3'-T_2AM, ^{15}N-3,5-T_2, 3,5-T_2AM, 3'-5'-T_2AM, T_1Ac, T_0Ac
Isoscience (King of Prussia, USA)
$^{13}C_6$-T_3, $^{13}C_6$-rT_3, $^{2}H_5$-T_4

Table 6: List of equipment used for all analyses

Device	Supplier
Autoklav Varioklav	Thermo Scientific (Waltham, USA)
DELFIA Plate Shaker	PerkinElmer (Rodgau-Jügesheim, Germany)
Electrophorese chamber	Bio-Rad Laboratories (Munich, Germany)
Dissecting set	FST Fine Science Tools GmbH (Heidelberg, Germany)
Eppendorf concentrator 5301	Eppendorf (Hamburg, Germany)
Magnetic stirrer Modell L32	A. Hartenstein (Würzburg, Germany)
Centrifuge 5417R	Eppendorf (Hamburg, Germany)
Microdismembrator Modell U	B. Braun Biotech (Melsungen, Germany)
Microwave M501	Whirlpool (Schorndorf, Germany)
iCyclerTM Thermal Cycler	Bio-Rad Laboratories (Munich, Germany)
Mithras LB 940 Luminometer	Berthold Technologies (Bad Wildbad, Germany)
Nanodrop ND-1000	PeqLab Biotechnologie (Erlangen, Germany)
pH meter inoLab Benchtop	WTW (Weilheim, Germany)
Pipettes Eppendorf research 0,1-2,5 µl, 0,5-10 µl, 10-100 µl, 100-1000 µl	Eppendorf (Hamburg, Germany)
Pipettor acu-jet pro	A. Hartenstein (Würzburg, Germany)
Hera cell incubator	Heraeus (Hanau, Germany)
Plate washer Hydroflex	Tecan (Crailsheim, Germany)
Vortex	A. Hartenstein (Würzburg, Germany)
Solid phase extraction station	Agilent Technologies (Böblingen, Germany)

Most of the used compounds only differ between the number and/ or position of an iodine atom or the deaminated/ decarboxylated side chain (Figure 13).

The diiodo-L-thyronines, all internal standards and all TAM except for T_4AM were dissolved in dimethyl sulfoxide (DMSO). T_0, monoiodo-L-thyronines, triiodo-L-thyronines and T_4AM were dissolved in DMSO-containing 100 µM hydrochloric acid. T_4 was dissolved in DMSO-containing 100 µM ammoniumhydoxide. All solutions were diluted with KH_2PO_4/K_2HPO_4-buffer (100 mM, pH 7.4) to a final concentration of less than 1‰ DMSO in all experiments. L-DOPA was always freshly prepared by dissolving it in KH_2PO_4/K_2HPO_4 buffer (100 mM, pH 7.4).

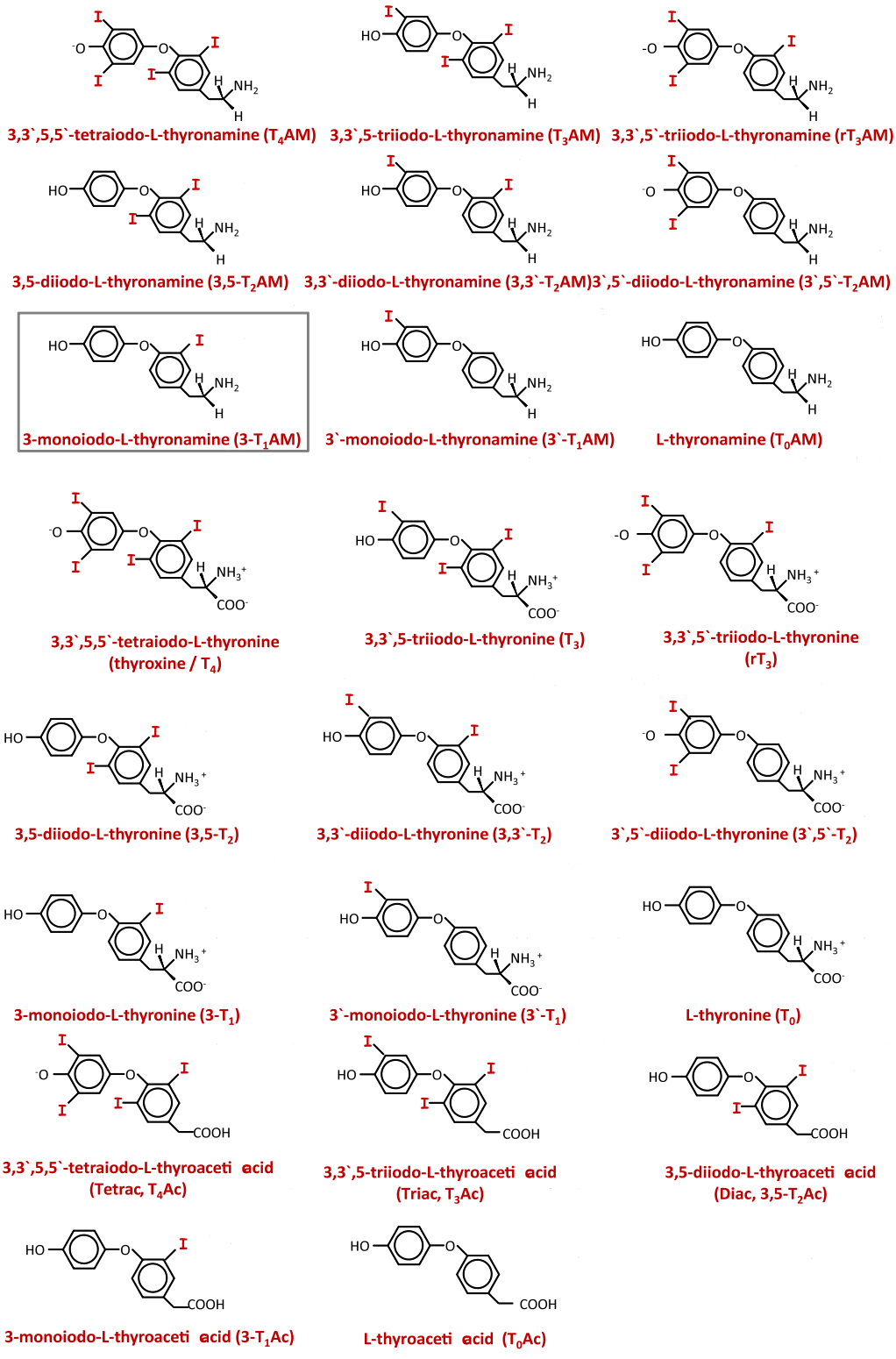


Figure 13: Chemical structures of 3-T_1AM related compounds.

All possible and available thyronamines, iodothyronines and thyroacetic acids are shown in comparison to the structure of 3-T_1AM (grey box).

Table 7: List of software used in this work

Software	Supplier
GraphPad Prism 5.01	GraphPad Software
MicroWin 2000	Berthold Technologies
MS Offce	Microsoft
Multicalc program	PerkinElmer
Analyst 1.5	AB SCIEX

Table 8: List of buffers used in this work

Buffer name	Buffer composition
Homogenization buffer	250 mM D(+) sucrose, 20 mM HEPES, 1 mM EDTA in H_2O, pH 7.4, stored at 4°C
Perfusion buffer	0.1 M Sodium potassium phosphate buffer, pH7.4
1 x TAE buffer	40 mM tris-acetate, 1 mM EDTA in ddH_2O, pH8.3, stored at RT
6 x DNA loading buffer	0.25% (v/v) saturated, aqueous bromphenol blue solution, 30% (v/v) glycerol in ddH_2O, stored at RT
Immunoassay buffer	50 mM Trizma®base, 154 mM NaCl, 20 µM DTPA, 0.01% Tween 40, 0.05% NaN_3, 0.05% bovine γ-globulin and 0.5% BSA in ddH_2O, filtration with 8 µm cellulose nitrate filter, pH 7.7 ,stored at 4°C
Coating buffer	50 mM Na_2HPO_4 in ddH_2O, pH 7.4, stored at room temperature (RT)
Enhancement solution	**100x Enhancement solution I** 100 mM thenoyltrifluoroacetone, 10 mM tri-n-octylphosphinoxide, 2% EtOH and 20% Triton®X-100 in ddH_2O, stored at 4°C **10x Enhancement solution II** 68 mM KHP and 6% acetic acid in ddH_2O, stored at 4°C **Working solution** 1% Enhancement solution I mixed with 10% Enhancement solution II, stored at RT, wrapped with aluminium foil
PBST	4 PBS tablets were dissolved in 1L ddH_2O and 0.01% Tween 40 were added, stored at 4°C
AADC incubation buffer	100 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.4, stored at RT

2.1 LC-MS/MS analysis

LC-MS/MS analysis of TH, TAM and TAc in biological samples were performed using a Shimadzu ultrafast liquid chromatograph (UFLC) system consisting of a controller unit, two pumps including a degasser (Shimadzu Scientific Instruments, Columbia, MD, USA) and an autosampler (CTC Analytics AG, Zwingen, Switzerland). Data processing was performed using Analyst version 1.5. Software .

The column oven and the autosampler were operated at 40°C and 10°C, respectively. Analytes were detected using a QTRAP®4000 triple-quadrupole tandem mass spectrometer (AB SCIEX, Darmstadt, Germany) equipped with a TurboIonSpray interface. Nitrogen served as nebulizer, curtain and collision gas. Because the analytes fall into different chemical classes, depending on ionizable groups including acids (TAc), bases (TAM), and zwitter ions (TH), it was necessary to develop two different methods. Although TH and TAM can be analyzed in the positive and negative (ESI) mode, the TAc were only detectable in the ESI negative mode. Since the highest sensitivity for TAM and TH was reached in the positive mode, a second negative ESI run was established to measure the TH, TAM and TAc in one sample.

The detection was performed in the selected reaction monitoring (SRM) mode. The device-specific mass spectrometric working parameters used to record the parent ion mass spectra and product ion tandem mass spectra were: TurboIonSpray voltage (IS): 5500 V, curtain gas (CUR): 30 psi, collision gas (CAD): 5 psi, nebulizer gas (Gas1): 60 psi, heater gas (Gas2): 50 psi, entrance potential (EP): 10 V, source temperature: 400°C, dwell time: 50 ms. Chromatographic separation was achieved using a Synergi Polar-RP 80-Å column (150 x 2 mm; Phenomenex, Aschaffenburg, Germany) and an analytical Guard Cartridge System (4.0 mm x 2.0 mm, Phenomenex, Aschaffenburg, Germany), using a gradient elution program at a flow rate of 300 µl/min. For HPLC separation, 0.6% acetic acid in 5% acetonitrile was used as the aqueous mobile phase (A), and 0.6% acetic acid in 95% acetonitrile was used as the organic mobile phase (B) (Figure 14).

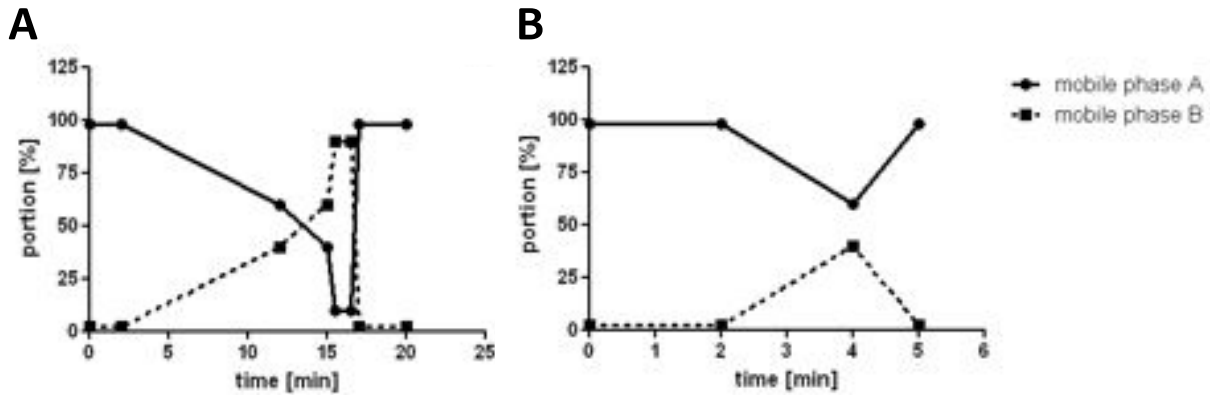


Figure 14: Parameters of the gradient elution program used for the chromatographic separation of TH/TAM (A) and L-DOPA/dopamine (B).

Mobile phase A: 0.6% acetic acid in 5% acetonitrile was used as aqueous phase, mobile phase B: 0.6% acetic acid in 95% acetonitrile was used as the organic phase. The figures show the portion of each mobile phase over the running time [min] of each method.

Analytes were diluted in 1% acetic acid in H_2O/acetonitrile (50:50 (v/v)) to obtain a 10 µM working solutions. This working solution was directly injected into the mass spectrometer at a flow rate of 10 µl/min using a Hamilton syringe device (Hamilton, Darmstadt, Germany). MRM transitions to be monitored and compound-dependent LC-MS/MS parameters are summarized in Table 9.

Table 9: Optimization of compound-specific mass spectrometric parameters.

Compound	(m/z)[a] Q1[b]	(m/z) Q3[c]	DP[d]	CE[e]	CXP[f]
Positive ESI mode					
T_0	274.1	257.0 215.1	81.0 81.0	17.0 27.0	48.0 14.0
T_1	400.0	382.9 354.0	86.0 86.0	21.0 25.0	12.0 10.0
T_2	525.8	479.8 151.9	101.0 101.0	29.0 125.0	14.0 26.0
T_3	651.8	605.7 479.0	116.0 116.0	29.0 51.0	18.0 24.0
T_4	777.7	731.6 350.9	50.0 50.0	33.0 53.0	22.0 10.0
T_0AM	230.1	213.1 109.1	76.0 76.0	19.0 35.0	14.0 18.0
T_1AM	356.0	339.0 212.1	91.0 91.0	19.0 27.0	10.0 14.0
T_2AM	481.9	464.8 338.0	101.0 101.0	21.0 25.0	6.0 10.0

T_3AM	607.8	590.9 210.1	116.0 116.0	23.0 61.0	20.0 16.0
T_4AM	733.7	716.6 151.9	126.0 126.0	29.0 127.0	22.0 26.0
DOPA	198.2	152.1 107.0	61.0 61.0	23.0 37.0	26.0 18.0
Dopamine	154.1	137.1 91.1	71.0 71.0	17.0 35.0	10.0 6.0
d_4-3-T_1AM	360.0	343.1 216.1	86.0 86.0	21.0 29.0	10.0 34.0
^{15}N-3,5-T_2	535.9	488.9 362.1	91.0 91.0	29.0 43.0	14.0 10.0
$^{13}C_6$-T_3/ $^{13}C_6$-rT_3	657.7	611.8 203.1	101.0 101.0	31.0 103.0	16.0 18.0
$^{2}H_5$-T_4	782.7	731.6 201.0	111.0 111.0	37.0 97.0	20.0 14.0
Negative ESI mode					
T_0	271.9	107.8	-80.0	-70.0	-5.0
T_1	397.8	126.8	-95.0	-36.0	-7.0
T_2	521.8	126.8	-85.0	-40.0	-7.0
T_3	649.7	126.8	-110.0	-60.0	-5.0
T_4	775.7	126.9	-105.0	-90.0	-5.0
T_0AM	227.9	107.9	-90.0	-30.0	-5.0
T_1AM	353.7	126.9	-80.0	-26.0	-7.0
T_2AM	479.9	126.9	-75.0	-30.0	-5.0
T_3AM	605.9	126.9	-105.0	-58.0	-5.0
T_4AM	731.7	126.9	-110.0	-86.0	-5.0
T_0Ac	242.8	126.7	-50.0	-22.0	-13.0
T_1Ac	368.9	126.7	-40.0	-14.0	-21.0
Diac	494.7	126.7	-35.0	-26.0	-7.0
Triac	620.7	126.7	-15.0	-58.0	-5.0
Tetrac	746.5	126.8	-90.0	-54.0	-11.0
d_4-3-T_1AM	357.9	126.9	-90.0	-26.0	-7.0
$^{13}C_6$-rT_3	655.8	126.8	-90.0	-90.0	-7.0
$^{13}C_6$-T_3	655.7	126.8	-90.0	-74.0	-7.0
$^{2}H_5$-T_4	780.7	126.8	-95.0	-88.0	-7.0

Legend:[a] (m/z): mass to charge ratio; [b] (m/z) Q1: m/z of parent ion in first quadrupole; [c] (m/z) Q3: m/z of most intensive product ion in third quadrupole; [d] DP: declustering potential (V); [e] collision energy (V); [f] collision cell exit potential (V).

2.2 Preanalytical extraction procedures

2.2.1 Liquid-liquid extraction procedure

Extraction of all TH and TAM from the same biological sample was performed using a liquid-liquid extraction (LLE) procedure as described before (Piehl, et al., 2008). Assays were performed on serum, tissue homogenates, cell culture incubation medium, and cell culture lysate. Approximately 0.1-0.3 g of the tissue was homogenized at 4°C in 0.2 ml of homogenization buffer using a dounce homogenisator (glass/glass). Briefly, enzymatic reactions were stopped by adding 0.1 volumes 100% acetic acid. Subsequently, internal standards were added to each vial. Acidified reaction mixtures were incubated at 37°C for 30 min. Proteins were precipitated by adding three volumes ice-cold acetone followed by an incubation at -20°C for 15 min. After centrifugation at 14.000 x g and 4°C for 5 min, supernatants were transferred into new Eppendorf tubes, acidified with 0.002 volumes 30% HCl, washed twice with 1 volume cyclohexane and then subjected to three subsequent extractions with 1.5 volumes ethyl acetate. Organic layers were combined and evaporated to dryness at 45°C. Residues were redissolved in 30 µl 1% acetic acid in H_2O/acetonitrile (90:10 (v/v)) and stored at -20°C until analysis by LC-MS/MS.

2.2.2 Urea-solid phase extraction procedure

Extraction of 3-T_1AM from serum was performed using a modified SPE procedure as described before (DeBarber, et al., 2008). Serum samples were spiked with IS. Three volumes 8 M urea were added for 30 min at 80°C to dissociate 3-T_1AM from binding proteins. Proteins were precipitated by addition of two volumes acetone, pH 4 (acidified with concentrated HCl). The samples were vortexed for 30 s and centrifuged at 14.000 x g for 5 min. The supernatant was removed and the samples were evaporated to dryness at 45°C. Residues were dissolved in 2 mL 100 mM phosphate buffer, pH 6, for loading onto 130 mg/3 mL cation-exchange SPE cartridges. The cartridges were preconditioned under positive pressure with air using 2 ml methanol (2 mL), de-ionized water (2 mL) and phosphate buffer (1.5 mL). Samples were loaded onto the column under gravity and were sequentially washed with water (2 mL). 3-T_1AM was eluted with 2% (v/v) ammonium hydroxide in methanol (2 mL, then 0.5 mL twice). The solvent was removed by evaporation and reconstituted in 30 µL 1% acetic acid in H_2O/acetonitrile (90:10 (v/v)) and stored at -20°C until analysis by LC-MS/MS.

2.2.3 Solid phase extraction procedure

The SPE procedure using HybridSPE® cartridge from Sigma Aldrich (Taufkirchen, Germany) was done according to the manufactures instructions. The technology utilizes a proprietary zirconia-coated particle, and exhibits a selective affinity towards phospholipids while remaining non-selective towards a range of basic, neutral and acidic compounds. Therefore, 200 µl human serum was spiked with IS and deproteinized by a precipitating agent consisting of 1% formic acid in acetonitrile (1:3 (v/v)). The samples were vortexed for 3 min and centrifuged at 3.000 x g for 5 min at RT. Precipitated protein was removed and the supernatant was transferred to a HybridSPE® cartridge. After applying vacuum, the resulting eluent was concentrated in an Eppendorf concentrator, reconstituted in 30 µL 1% acetic acid in H_2O/acetonitrile (90:10 (v/v)) and stored at -20°C prior to LC-MS/MS analysis.

2.3 Cell culture experiments

2.3.1 Cell culture

The rat thyroid follicular cell line FRTL-5 was used as model systems to study cellular TH and TAM uptake. Cells were cultured in COON's-F12 supplemented with 10% FCS, 1% Penicillin/Streptomycin, 6H-Supplement (10 ng/ml Glycyl-L-histidyl-L-lysin Acetate, 10 nM Hydrocortisone, 10 µg/ml bovine Insulin, 10 ng/ml Somatostatin, 5 µg/ml apo-Transferrin, 100 µU/ml TSH.) and 100 nM Na_2SeO_3 to allow for optimal expression of DIO selenoproteins.
Cells were propagated in an incubator at 37°C, 95% humidity and at 5% CO_2 (now referred as standard conditions). Medium was exchanged at 3-day intervals and cells were passaged on a weekly basis, using a 1:3 splitting ratio. In order to prepare TH treatments, cells were counted using a haemocytometer and seeded at a concentration of 5 x 10^4 cells per 6-well and grown to 70% confluence. After 24 h, all medium was removed and cells were washed twice with PBS. Cells were stimulated with the indicated concentrations of various TH metabolites in serum-free medium.

2.3.2 RNA extraction and cDNA synthesis

RNA extraction was performed using RNAEasy Mini Kit according to the manufacturer`s instructions. (Qiagen, Hilden, D). Finally, the RNA-pellet was dissolved in 30 µl HPLC H_2O. For determination of RNA concentration and purity, the extinction of 1 µl RNA was measured at

260 nm using the UV/Vis spectralphotometer Nanodrop ND-1000. cDNA was subsequently synthesized from 500 ng RNA using iScript kit (Bio-Rad, Hercules, USA) according to the manufacturer`s instructions. Incubations were performed in a thermal cycler.

2.3.3 Polymerase chain reaction (PCR)

In preliminary experiments, annealing temperatures between 58°C and 62°C were tested for each gene in order to optimize the sensitivity, specificity and efficiency of the reactions. The best conditions for the PCR were determined after a series of test-PCRs with a variation of distinct factors. The best conditions are displayed in Table 10. Annealing temperatures were chosen according to the used gene specific primers.

Table 10: Optimized thermal cycler conditions for PCR analyses.

Event	Temperature [°C]	Time [sec]	Repetition
Primery denaturation	94	60	
Denaturation	94	30	40x
Annealing	58 /60/ 62	30	
Elongation	68	30	
Final elongation	68	150	
Storage	8	∞	

For the PCR reaction, the KAPAHiFi-polymerase (KAPA Biosystems) was used. The PCR-sample had a final volume of 25 µl (10 µl mastermix (containing buffer, 10 mM dNTPs and polymerase), 1 µl reverse primer (10 µM), 1 µl forward primer (10 µM), 2 µl template DNA, 11 µl HPLC H_2O). Primers sequences, fragment lengths and annealing temperatures are listed in Table 11.

2.3.4 Gel electrophoresis of PCR products

The sizes of the PCR products were estimated by the primer design and confirmed by horizontal agarose gel electrophoresis. Therefore, 1.5% (w/v) Agarose was dissolved by boiling in 1 x TAE buffer. 5 µl ethidium bromide solution/100 ml gel were added, prior to the solidification of the gel. For sample preparation, 10 µl PCR product and 3 µl 6 x DNA loading buffer

were adjusted to a final volume of 18 µl by adding 5 µl ddH_2O. 15 µl of each sample were loaded onto the gel. To estimate the sizes of the PCR products, 5 µl molecular size marker (Gene Ruler^{TM} 1 kb DNA ladder plus (Fermentas, St. Leon-Rot, Germany), were also electrophorized on each gel. Electrophoresis was performed for 90 min (100mV and 400mA running conditions) and using 1 x TAE buffer as running buffer.

Table 11: List of rat gene-specific primer pairs used for PCR analyses including the respective nucleotide sequences, PCR fragment sizes and annealing temperature.

Gene	fw/ rev	Nucleotide sequence (5' → 3')	Fragment [bp]	Annealing [°C]
Dio1	fw	TTAAGAACAACGTGGACATCAGG	197	60
	rev	GGTTTACCCTTGTAGCAGATCCT		
Dio2	fw	GCGACCTGACCACCTTTTACTAG	191	60
	rev	GCAGCACATCGGTCCTCTTG		
NIS	fw	GGTGTCATCAGTGGGCCTCTA	269	60
	rev	CCCGTGTCCATTCCAGAACTG		
Pendrin (SLC26A4)	fw	GCCCAGTGGTAACGGAAGTA	217	60
	rev	CTGTGAGACCAGCACTTGGA		
Tg	fw	CCGGATATTGCAGAGACGAT	128	60
	rev	GGCAGCTTGGGATATATGGA		
Duox1	fw	GCCCTTGAGAGAACCCTACC	158	60
	rev	AGCCTGGTGTTTCCACACTC		
Duox2	fw	CTGGACAGAGCCCTAGCAAC	129	60
	rev	CCAGCTGTCCTCCAGAGAAG		
TPO	fw	TTGGATCTGGCATCACTGAACTT	149	60
	rev	ATCTTGTTGACCATGCTTCTGTTG		
MCT8 (SLC16A2)	fw	CCTCGCTATGGGCATGATCT	202	62
	rev	TGGTTGAAAGGCGAATGAGC		
THRα1	fw	GACAGGGCGACAAATGAACT	239	58
	rev	GGCCTGAAGGGAAATCTAGG		
THRα2	fw	ATGCCTGGCAATACCTTGTC	197	58
	rev	GCACTGGGCTACAGACATGA		
THRβ1	fw	GCTAGCCAAGAGGAAGCTGA	208	58
	rev	GGGTGCTTGTCCAATGTCTT		
TAAR1	fw	TGAGGAGCAGTATCACAATCAG	181	58
	rev	ATTTGCACGATTAATTGACCTC		

2.4 Development of a monoclonal antibody-based immunoassay

2.4.1 Conjugation of 3-T_1AM to BSA and HRP

To couple 3-T_1AM to BSA, 10 mg/ml BSA (bovine serum albumin) and EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) were dissolved in demineralized water. 4 mg/ml 3-T_1AM was dissolved in DMSO and diluted 1:25 in 0.1 M MES (2-(N-morpholino)ethanesulfonic acid) buffer (pH 5.0). BSA, EDC as a coupling reagent and 3-T_1AM were combined in a reaction tube wrapped in aluminum foil, to form the 3-T_1AM-BSA conjugate within 120 min of shaking at RT (Figure 15). The conjugate was dialyzed overnight at 4°C against PBS (pH 7.4) using Slide-A-Lyzer Dialysis Cassettes (20K molecular-weight cutoff, Pierce Chemical Co., Rockford, USA), sterile filtered and stored frozen in aliquots at -20°C.

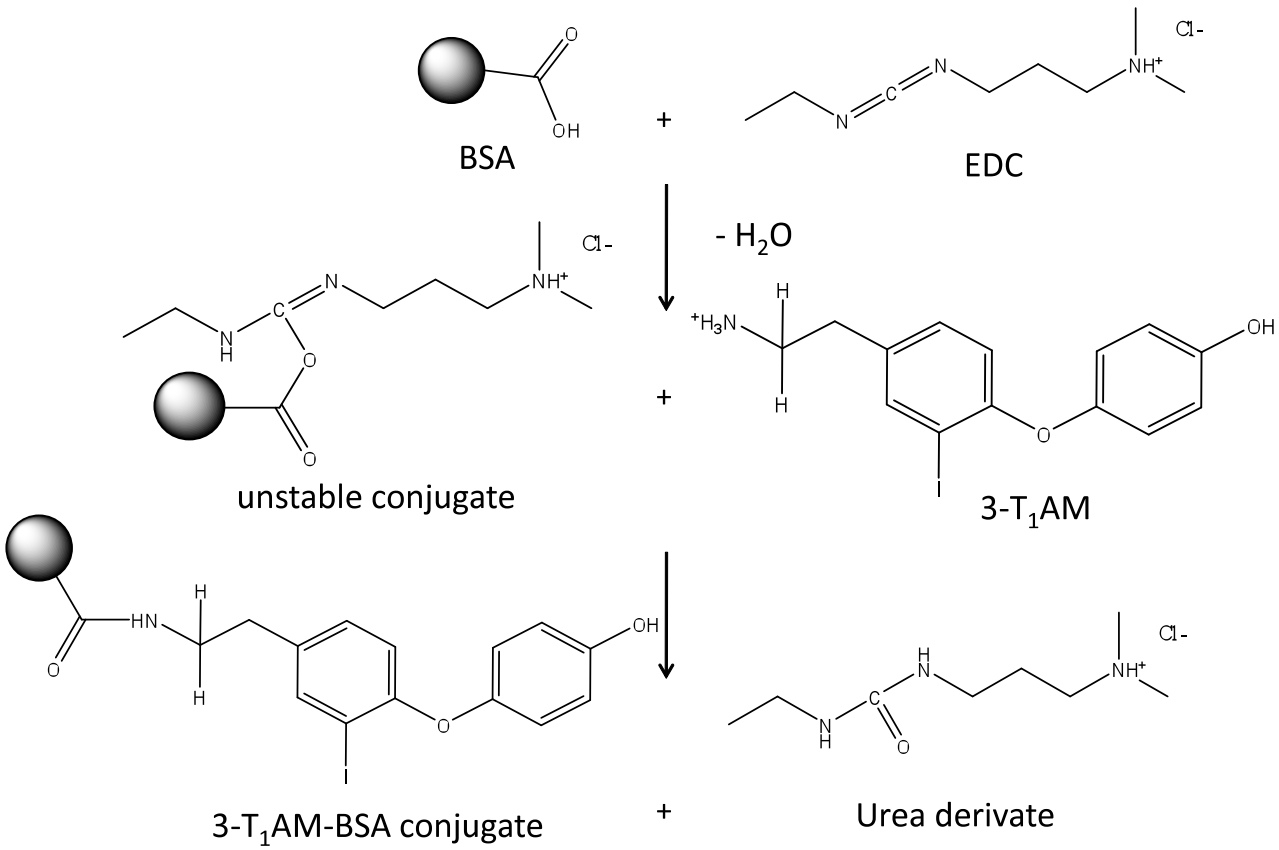


Figure 15: Principle of the 3-T_1AM conjugation to BSA to form the hapten for immunization.

EDC was used for coupling 3-T_1AM to the carrier protein BSA. EDC reacts first with a carboxyl group of BSA and forms an unstable amine-reactive intermediate that quickly reacts with the amino group of 3-T_1AM to form the amide bond of the 3-T_1AM-BSA conjugate and release an urea by-product.

The same procedure was used to couple 3-T_1AM to HRP (horse radish peroxidase) to form a labeled 3-T_1AM-HRP complex for construction of the immunoassay. The protein concentration of the dialyzed 3-T_1AM-HRP was measured to calculate the final 3-T_1AM-HRP concentration. The 3-T_1AM-HRP conjugate was sterile filtered and stored in aliquots at 4°C.

2.4.2 Immunization of mice with 3-T_1AM-BSA conjugate

Three two-month-old female BALB/c mice (Jackson Laboratory, Maine, USA) were immunized with BSA-coupled 3-T_1AM. The antigen was dissolved in TiterMax®Gold adjuvant according to manufacturer's recommendations (Sigma Aldrich, Taufkirchen, Germany).

For priming, 50 µg of antigen was injected at four local points subcutaneously in each mouse. Immunized mice were boosted twice using 25 µg antigen in TiterMax®Gold. Blood samples were obtained from mice for measurement of serum antibodies. The anti-3-T_1AM antibody titer within the serum of immunized mice was monitored using the same methods as used for the screening of hybridoma supernatants (see 2.4.9).The two last injections (25 µg antigen in PBS) were given intraperitoneally 3 and 2 days before harvesting the spleen from the mouse with highest antibody titer. Therefore, the immunized mouse was killed by cervical dislocation and the spleen was removed (Figure 16).

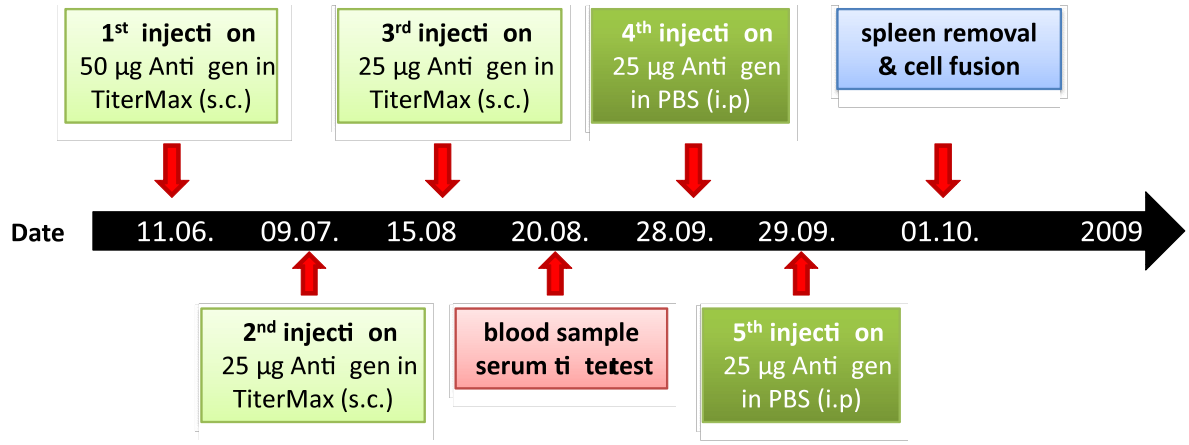


Figure 16: Scheme of the mouse immunization protocol.

2.4.3 Preparation of lymphocyte cell suspension

The mouse spleen will serve as a source of lymphocyte cells for fusion with mouse myeloma cells yielding hybridoma cells (Figure 5). The spleen was removed and placed into a tube containing 10 ml of cold, sterile DMEM (Dulbecco`s Motified Eagle`s Medium). The removed spleen was transferred into a petri dish with 10 ml DMEM without serum and minced to free the lymphocytes. The resuspended cells were counted using a haemocytometer to calculate the number of viable lymphocytes/ml.

2.4.4 Preparation of a mouse myeloma cell line

The mouse myeloma cell line PAI was used for hybridoma formation with spleen lymphocytes. PAI cells lost their ability to secret antibodies (non-secretor) and were originally isolated from a B-lymphocyte tumor of a BALB /c mouse strain. Standard sterile cell culture techniques were used to prepare the PAI suspension culture for cell fusion. PAI cells were cultured in DMEM with 4.5 g glucose/l containing 4 mM L-glutamine, 100 U penicillin, 100 µg/ml streptomycin, 1 µg/ml fungizon, 10% horse serum (HS) and 1 mM pyruvate (HT medium) in an incubator at 37°C, 95% humidity and at 10% CO_2. Cells were fed every day and subcultured in the log phase under sterile conditions. Therefore, 10 ml of cell suspension was put into a fresh cell culture flask containing 10 ml of new, pre-warmed (37°C) medium. Cells were placed into an incubator under standard conditions. To determine the number of viable myeloma cells/ml the haemocytometer were used.

2.4.5 Preparation of macrophage cells

On the day of cell fusion, macrophage cells of an untreated BALB/c mouse were prepared as feeder cells for cell fusion. Feeder cells supply growth factors that promote growth of the hybridoma cells. Hence, 5 ml sterile cold DMEM medium was injected into the peritoneal cavity of the mouse. The abdomen of the mouse was gently massaged and the macrophage cell suspension from the peritoneal cavity was drained. Macrophage cells were diluted in DMEM medium up to 10 ml, centrifuged for 5 min at 1,000 rpm and 4°C. After removing the supernatant, cells were resuspended in 100 ml DMEM with 4.5 g glucose/l containing 4 mM L-glutamine, 100 U penicillin, 100 µg/ml streptomycin, 1 µg/ml fungizon, 20% HS and 1 mM pyruvate (HAT medium). Ten 96-well plates were prepared with 4-5x10^3 feeder cells/ 100 µl HAT medium/ well and placed into an incubator under standard conditions.

2.4.6 Cell fusion using Polyethylene Glycol 1500

Antibody-producing spleen cells have a limited life span. The fusion with immortal lymphatic myeloma cells results in an antibody-producing hybridoma with unlimited growth. The HAT medium allows only the fused cells to survive in culture. Fusion is accomplished by incubating freshly harvested spleen cells with myeloma cells in 50% Polyethylene Glycol (PEG) 1500 (PEG 1500 (w/v) in 75 mM Hepes, pH 8.0, Roche Diagnostics GmbH, Penzberg, Germany). PEG 1500 is a polyether substance that causes cell membranes to fuse. Single spleen cells from the immunized mouse are mixed with the previously prepared myeloma cells (2 x 10^7 cells) in 40 ml cold DMEM without serum to create a 5:1 ratio lymphocytes/myeloma cells. Cells were centrifuged for 5 min at 1,000 rpm and 4°C. After removing the supernatant, cells were resuspended in 40 ml fresh, cold DMEM and were centrifuged again for 5 min at 1,000 rpm and 4°C. The resulting supernatant was discarded very carefully and the remaining pellet was gently dispensed. Afterwards, 1 ml sterile pre-warmed 50% PEG 1500 solution was added to the cell mixture over the period of 1 min under softly agitation. The cell suspension was placed into a 37°C warm water bath for 1 min, before 1 ml pre-warmed DMEM was added over the period of 1 min under soft agitation. After another minute in the water bath, 19 ml pre-warmed DMEM was added over the period of 5 min and 30 ml over the period of 1 min under slow shaking. Following a further 5 min incubation period in the water bath, the cells were centrifuged for 5 min at 1,000 rpm and 20°C. The supernatant was discarded and the remaining pellet was gently dispensed in 50 ml pre-warmed HAT medium. The first 10 ml were added over the period of 2 min followed by the remaining 40 ml within 5 min. The cell solution was distributed to the ten 96-well plates (50 µl/well) containing the feeder cells and placed in an incubator. The cells were fed with 50 µl fresh HAT medium after 4-5 days. After 7 days 100 µl conditioned medium was replaced by 100 µl of fresh HAT DMEM medium. Finally, cells were subcultured in HT DMEM medium every 4-5 days by replacing 100 µl conditioned medium with fresh medium. The supernatant from all hybrid cells was screened for production of the desired antibody (see 2.4.9).

2.4.7 Expansion of the antibody-producing cells

Positive clones were transferred into larger volumes of medium to ensure vigorous cell growth and cell division. Therefore, promising candidate cells in one 96-well plate were gradually transferred into bigger plates before the desired hybridoma cell clone is cloned by

"limiting dilution" (see 2.5.8) and expanded in a high density cell culture system (Mini-PERM, Heraeus (Hanau, Germany)).

2.4.8 Cloning of hybridoma cells by "Limiting Dilution"

Cloning by limiting dilution is a procedure for separating cells in cell culture. If one cell per well remains viable and proliferates, then an isolated clone will arise which produces only one specific antibody. A suspension of 4-5x10^3 feeder cells/ 100 µl HAT medium per 96-well plate was prepared. Approximately 0.2 ml of the lymphocyte cell suspension was placed into each well of a 96-well plate and incubated under standard conditions. 25 µl of the hybridoma cell solution from a 24-well plate were dispensed into all 12 wells of the first row of a 96-well plate. After softly mixing, 50 µl of each well were taken to the next 12 wells of the second row. This procedure was repeated until the last 12 well in the last row. The plates were incubated at 37°C in 10% CO_2 for 4-7 days without changing the medium. The supernatant from all wells that contain one hybridoma clone/well was screened for production of the desired antibody and the antibody specificity was tested by a screening assay (see 2.5.9). A second and third cloning by limiting dilution was performed to insure the production of a true clone.

2.4.9 Screening of hybridoma supernatant and mouse serum

96-well flat-bottom high binding microtiter plates (Maxisorp plates, Nunc, Roskilde, Denmark) were coated with 100 ng/100 µl 3-T_1AM-KLH, 3-T_1AM-BSA and BSA diluted in Na_2HPO_4 buffer (pH 7.4) and incubated overnight at 4°C. After washing the plates 3 times with PBS containing 0.05% Tween-20 (PBST) using a Hydroflex® Strip plate washer, serum dilutions (1:2,000; 1:20,000; 1:200,000) from immunized mice or hybridoma supernatant (diluted 1:4 in assay buffer) were added to the plates (100 µL/ well) for 1 h at RT under agitation. Plates were washed again 3 times with PBST before a rabbit- anti-mouse antibody (10 ng/100 µl) diluted in assay buffer was added to the plates for 1 h at RT under agitation on a DELFIA ® Plate Shaker. After washing 3 times with PBST, 10 ng Europium labeled streptavidin per well in 100 µl diluted in assay buffer was added and incubated for 30 min at RT under agitation on a DELFIA ® Plate Shaker. After washing the plates 6 times with PBST, 0.2 ml enhancement solution was added to each well and incubated for 10 min on a DELFIA® Plate Shaker. The fluorescence signal was then read by VICTOR3™ Multilabel Counter (PerkinElmer, Waltham, USA). Concentrations were automatically calculated according to a standard curve fitting by the Multicalc program.

2.4.10 Freezing and thawing hybridoma cells

Freezing of hybridoma cells was done using cells growing in a 24-well plate. 1 ml of the cell solution was transferred into a 15 ml falcon tube and centrifuged for 5 min at 1,000 rpm and RT. The supernatant was transferred into a 2 ml Eppendorf tube and 5% NaN_3 was added before the supernatants were stored at 4°C. The remaining pellet was gently dispensed in 0.5 ml freezing medium (50% (v/v) HS, 40% DMEM, 10% DMSO). Aliquots of the cell solution were transferred into cryovials, slowly frozen and stored in liquid nitrogen. Thawing of hybridomas was done very quickly by placing the frozen aliquot into the 37°C pre-warmed water bath until the cells are thawed. Cells were pipetted out into a new flask containing HAT medium. To wash out the DMSO-containing freezing medium, cells were centrifuged for 5 min at 1,000 rpm and RT before the cells were placed in fresh HAT medium.

2.4.11 Isotyping and purification of MAb

MAb were isotyped using a mouse MAb immunoPure Monoclonal Antibody Isotyping Kit (Pierce Chemical Co., Rockford, USA) according to the manufacturer's recommendations. The MAb were purified on a rProtein A Sepharose column with different conditions according to the isotypes of MAb and dialyzed against PBS using Slide-A-Lyzer Dialysis Cassettes overnight at 4°C. The optical density of the MAb was measured to calculate the final MAb concentration. The MAb was sterile filtered and stored in aliquots at 4°C.

2.4.12 3-T_1AM chemiluminescent immunoassay for human serum

White 96-well flat-bottom microtiter plates (Maxisorp plates, Nunc, Roskilde, Denmark) were coated overnight at 4°C with a rabbit-anti-mouse antibody (100 ng/0,1 ml per well). The plates were washed 3 times with PBST. 25 µl 3-T_1AM standard samples (0-500 nM) diluted in T_3/T_4 deficient serum or 7% BSA-PBST, respectively, control sera and serum samples were pipetted in duplicate into wells together with 25 µl HRP-labeled 3-T_1AM (10 ng) diluted in 0.5% BSA-PBST. 50 µl of MAb 9C5 (2 ng) diluted in 0.5% BSA-PBST was added before the plates were incubated at RT under agitation on a plate shaker for 90 min. After a final washing step (6 times with PBST), 0.1 ml luminol-based substrate solution was added to each well and incubated for 10 min on a plate shaker. The chemiluminescent signal was then analyzed by a multilabel counter and concentrations were automatically calculated according to the standard curve.

2.4.13 3-T_1AM chemiluminescent immunoassay for murine serum

White 96-well flat-bottom microtiter plates (Maxisorp plates, Nunc, Roskilde, Denmark) were coated overnight at 4°C with the MAb 9C5 (40 ng/0,1 ml per well). The plates were washed 3 times with PBS before 150 µl Liquid plate sealer®/ well (Candor Bioscience, Wangen im Allgäu, Germany) were added to the plate. After 1 h incubation, Liquid plate sealer® was discarded and the plate was dried overnight at 37°C. Afterwards, 25 µl 3-T_1AM standard samples (0-500 nM) diluted in 7% BSA-PBST and serum samples were pipetted in duplicate into the wells of the coated 96-well plate together with 75 µl HRP-labeled 3-T_1AM (10 ng) diluted in 0,5% BSA-PBST. The plates were incubated at RT under agitation on a plate shaker for 90 min. After a final washing step (6 times with PBST), 0.1 ml luminol-based substrate solution was added to each well and incubated for 10 min on a plate shaker. The chemiluminescent signal was then recorded by a multilabel counter and concentrations were calculated using the EXCEL software according to the standard curve.

2.4.14 Immunoprecipitation of 3-T_1AM

For the Immunoprecipitation experiment, the MAb 9C5 was coupled to magnetic beads. Therefore, 100 µl (50%) Sepharose4Fast Flow (1 mg/ml) was incubated with the MAb 9C5 in a 1.5 ml tube. The beads were washed twice with 1 ml PBST. After centrifugation at 6,000 rpm for 2 min, the supernatant was discarded carefully. 200 µl PBST were added to the beads and the beads were aliquoted in 2 x 100 µl in 1.5 ml Eppendorf tubes. 1 ml serum or 7% BSA-PBST were spiked with 100 nM 3-T_1AM and 100 µl MAb beads were added to the tube. The tube was incubated for 3 h under rotation at RT and afterwards over night at 4°C. Next day, the tube was again incubated for 1 h under rotation at RT. Afterwards, the samples were centrifuged at 6,000 rpm for 2 min and washed 3 times with PBST. The supernatant was discarded carefully and 100 µl 0.1 M sodium acetic acid (pH 3) were added to the pellet. The samples were shaken for 2 min at RT and finally centrifuged at 6,000 rpm and RT for 2 min. The supernatants were evaporated to dryness using an Eppendorf concentrator and resuspended in 30 µl 1% acetic acid in H_2O/acetonitrile (90:10 (v/v)) and stored at -20°C prior to LC-MS/MS analysis.

2.5 AADC experiments

2.5.1 AADC expression and purification

The expression and purification of human, recombinant AADC was done by Dr. Mariarita Bertoldi (University of Verona, Italy) and is published in Hoefig, et al. (Hoefig, et al., 2012).

2.5.2 AADC reaction *in vitro*

All assays were performed with purified, recombinant human AADC. Heat-inactivated AADC (boiled for 5 min at 95°C) was used as a negative control in all experiments. The incubation with the natural substrate, L-DOPA served as a positive control. 1 µM L-DOPA was incubated with 0.5 µM AADC preparation in KH_2PO_4/K_2HPO_4-buffer (100 mM, pH 6.8) and 0.6 mM PLP for 30 min at 37°C. Using the same incubation conditions, this experiment was repeated for all TH.

Thereafter, the impact of different factors on the decarboxylation of rT_3 by AADC was tested varying buffer pH, incubation time, and temperature. To investigate the impact of the incubation time, 0.5 µM AADC preparation was pre-incubated with PLP (0.6 mM) in KH_2PO_4/K_2HPO_4-buffer (100 mM, pH 7.2) for 0.5 h at 37 °C. After adding 100 nM rT_3, the reaction mix was incubated at 37°C. Different samples were transferred to new tubes in 2h intervals from 0 h-18 h. To study the effect of the buffer pH, 100 mM KH_2PO_4/K_2HPO_4 (pH: 5.0, 6.0, 6.8, 7.2, 7.6, 8.0 and 8.8), and 0.5 µM AADC preparation was pre-incubated with 0.6 mM PLP for 0.5 h at 37°C. Afterwards, 100 nM rT_3 was added to the reaction mix followed by an incubation for 30 min at 37°C. To study the impact of the incubation temperature, buffer containing 100 mM KH_2PO_4/K_2HPO_4 (pH 6.8) was used and the incubation temperature was varied between 35°C and 43°C for 30 min.

2.5.3 Extraction of TH and TAM analytes from AADC preparation

Enzymatic reactions were stopped by heating the samples for 5 min up to 95°C. After cooling down, proteins were precipitated by adding 0.1 volume 100% trichloric acid and removed by centrifugation at 14,000 rpm for 5 min at RT. Supernatants were transferred to new Eppendorf tubes and concentrated by drying at 30°C using an Eppendorf concentrator. The residues were dissolved in 30 µl 1% acetic acid in H_2O/acetonitrile (90:10 (v/v)) and stored at -20°C until LC-MS/MS analysis.

2.6 Clinical samples

As internal control samples, we used the commercially available standard serum Lypho-check® as a positive control and T_4/T_3 deficient serum as a negative control.

Serum from 13 healthy volunteers (nine females and four males) was used to validate the assay and to study the normal concentration range of 3-T_1AM.

Ten patients with pituitary insufficiency (seven female, three male patients, age 28-57 years, with complete anterior pituitary deficiency (panhypopituitarism) in all but two patients where the corticotropic axis was preserved) under full T_4 replacement therapy and after six days of L-T_4 withdrawal were studied. Furthermore, 105 thyroid cancer patients on TSH suppressive L-T_4 therapy (80 female and 25 male patients, age 18-84 years, 77 with papillary, 21 with follicular, three with Hurthle cell and four with medullary thyroid carcinomas) were examined.

Sera from both clinical cohorts were obtained from the Department of Endocrinology, Christie Hospital, Manchester United Kingdom (Prof. Dr. med. Georg Brabant). All patients provided their informed consent and studies were approved by the local Ethical Committee.

Plasma from four patients with biochemically confirmed AADC deficiency were obtained from the Zentrum für Kinder- und Jugendmedizin der Universität Heidelberg (Professor Dr. med. Georg Hoffmann). The study was approved by the local Ethical Committee.

2.7 Animal organs

Organs from male, adult, euthyroid C57Bl/6 wild type mice were kindly provided by PD Dr. Ulrich Schweizer (Institut für Experimentelle Endokrinologie, Charité - Universitätsmedizin Berlin, Germany).

2.8 Statistical analysis

GraphPad Prism 5 software was used for all computations with tests indicated in the figure legends. Statistical significance was defined as $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

3 Results

3.1 LC-MS/MS Analysis of thyroid hormones and thyronamines

As the nine possible TH metabolites differ only in the number and/or position of iodine atoms and cover a broad serum concentration range (10^{-12} M (pM) to 10^{-9} M (nM)), their distinction and quantification by immunological methods is cumbersome.

Several publications for the measurements of TH in serum and tissue using LC-MS/MS exist, but only one publication by Piehl, et al. (Piehl, et al., 2008) describes the simultaneous detection of eight TH and TAM in one analytical run. Dr. Piehl established a sensitive and robust SRM-based LC-MS/MS method using the 3200® triple-quadrupole tandem mass spectrometer (AB SCIEX) at the Bundesinstitut für Risikobewertung (BfR). Thereby, both 3,3`-$T_2$AM and 3`,5`-$T_2$AM as well as 3,3`-T_2 and 3`,5`-T_2 were separated sufficiently to permit an unequivocal qualitative identification of each analyte. However, only an incomplete baseline peak separation was achieved for some analytes by the chromatographic method developed.

In this project, the LC-MS/MS analyses were performed using a Shimadzu UFLC system and a more sensitive QTRAP®4000 triple-quadrupole tandem mass spectrometer at the Interdisziplinäres Endokrinologisches Speziallabor (IESL) (Charité-Universiätsmedizin, Berlin). Therefore, the published LC-MS/MS method by Piehl, et al. for the simultaneous detection of TH and TAM (Piehl, et al., 2008) in a single sample needed to be optimized concerning both the HPLC and the MS/MS conditions. The HPLC running time was reduced from 30 min to 20 min without a loss of base line peak separation. The compound-specific and device-specific mass spectrometric parameters were optimized for the used method. To provide additional information and to correctly identify the individual analytes, a second MRM transition for all compounds (1st transition for quantification, 2nd transition for confirmation) was recorded (Table 9). Since the offline extraction recovery, the chromatographic retention time and the ESI ionization in the mass spectrometry differ between all TH and TAM, five important deuterated internal standards: d_5-T_4, $^{13}C_6$-T_3, $^{13}C_6$-rT_3, ^{15}N-T_2, d_4-T_1AM) were recorded to improve the accuracy of the TH and TAM quantification (Figure 17). Adding the correct deuterated IS to the sample containing the desired analyte should correct for any matrix effect.

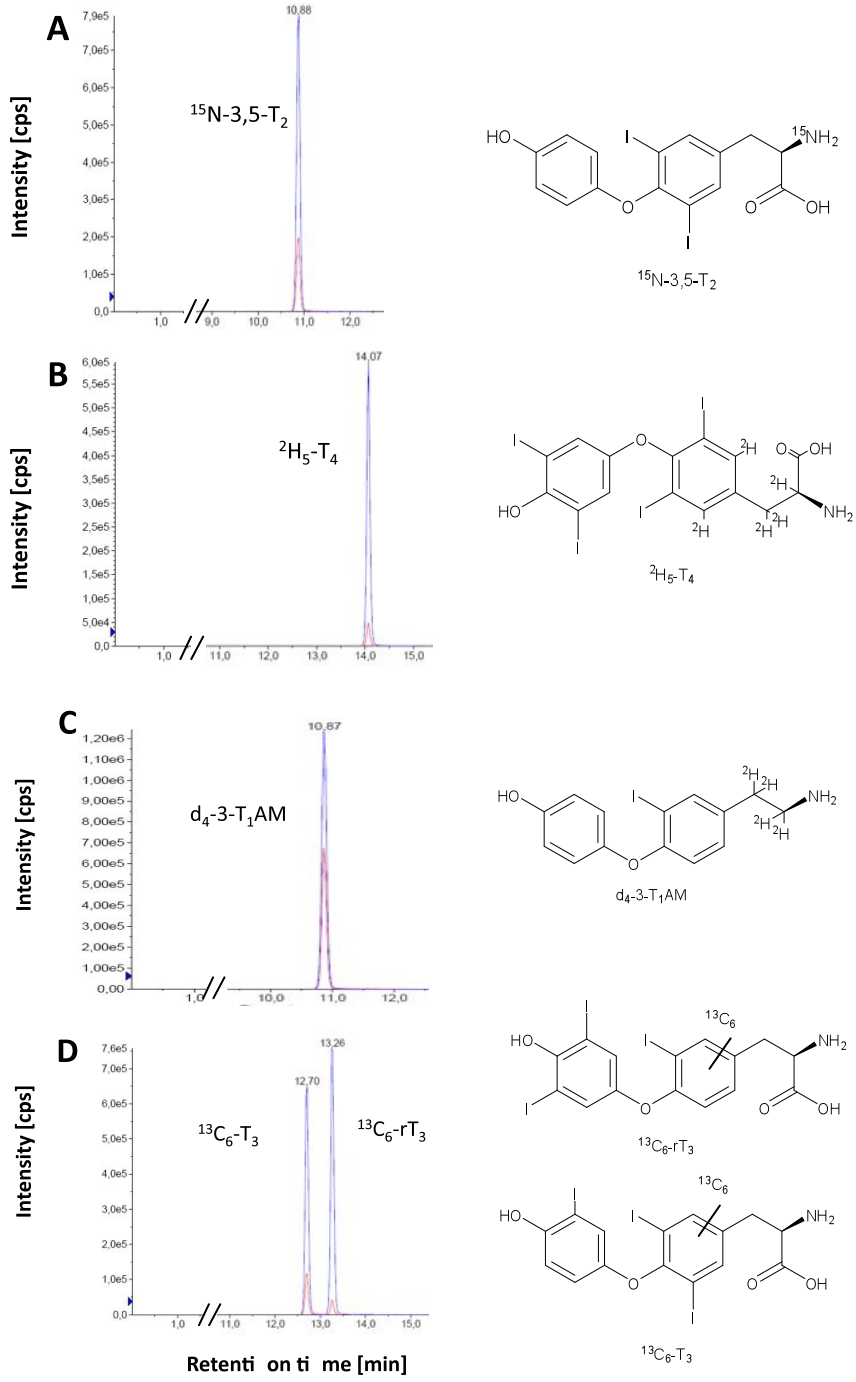


Figure 17: Representative chromatograms (two transitions per compound, blue and red traces) of the QTRAP®4000 LC-MS/MS analysis of all deuterated internal standards (^{15}N-T$_2$ (A), ^{2}H$_5$-T$_4$ (B), d$_4$-3-T$_1$AM (C), ^{13}C$_6$-T$_3$, ^{13}C$_6$-rT$_3$ (D)).

A key issue in LC-MS/MS analysis is the preanalytical workup or extraction of samples if these cannot be directly applied on the HPLC separation system because of analytical reasons, contamination or matrix interference. However, the preanalytical workup may also be of advantage if analytes are hereby enriched in the sample and/or contaminants removed, resulting in better signal to noise ratios. As TH and TAM concentrations in serum and biological samples span several orders of magnitude ranging from lower pM to higher nM concentrations, Piehl, et al. developed a LLE based method for the isolation of TH and TAM from mouse liver homogenates and cell culture extracts (Piehl, et al., 2008).

3.1.1 Liquid-liquid extraction for isolation of TH and TAM from serum

The published LLE procedure was tested for the isolation of TH and TAM from human serum. Figure 18 shows a representative chromatogram for the LLE and LC-MS/MS analysis of 200 µl human serum. The intensity of the T_4 and T_3 peaks were above the limit of quantification (LOQ), the other metabolites were below the limit of detection (LOD) (rT_3) or completely absent (3,5-T_2 and 3-T_1AM). The measurement of more than the classical TH, T_4 and T_3, was not achieved with this preanalytical extraction. However, exogenous 3-T_1AM or other TH metabolites added in serum are detectable confirming the accuracy of the LC-MS/MS measurement.

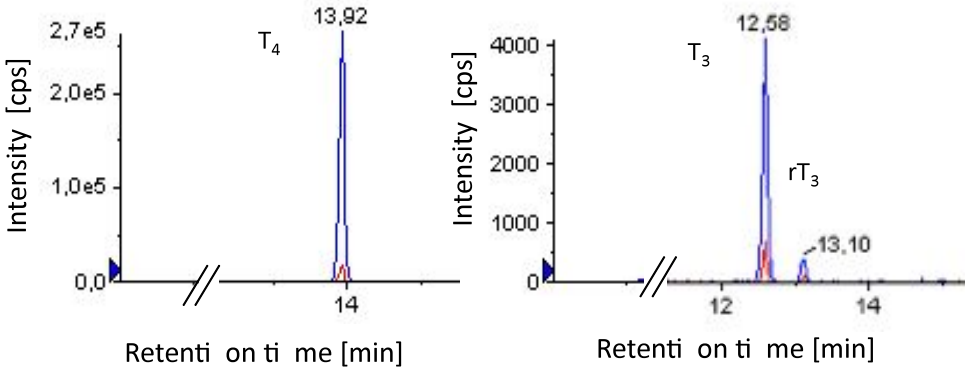


Figure 18: Representative chromatograms (two transitions per molecule) for the liquid-liquid extraction and QTRAP®4000 LC-MS/MS analysis of 200 µl human serum.

T_4 and T_3 are above the LOQ and rT_3 is below the LOD.

3.1.2 Isolation of TH and TAM from tissue samples

As the published LLE procedure was also used for liver homogenates, further mouse tissues were analyzed. Three mice (C57Bl/6) were perfused with potassium phosphate buffer (pH 7.4) and most organs were extracted to determine endogenous TH and TAM profiles. In several perfused organs T_4, T_3, rT_3, 3,5-T_2, and 3-T_1AM were detected. A qualitative overview of TH and TAM profiles in perfused mice organs is given in Table 12. In every tested organ T_4, T_3, 3,5-T_2 and 3-T_1AM could be detected. However, rT_3 was only detected in testis and the adrenal gland.

Table 12: Qualitative overview of mouse organs containing TH metabolites.

Organ	T_4	T_3	rT_3	3,5-T_2	3-T_1AM
cortex	xxx	xxx		xxx	xxx
cerebellum	xxx	xxx		xxx	xxx
pituitary	xxx	xxx		xxx	xxx
hypothalamus	xxx	xxx		xxx	xxx
testis	xxx	xxx	xxx	xxx	xxx
duodenum	xxx	xxx		xxx	xxx
ileum	xxx	xxx		xxx	xxx
caecum	xxx	xxx		xxx	xxx
jejunum	xxx	xxx		xxx	xxx
stomach	xxx	xxx		xxx	xxx
kidney	xxx	xxx		x	xx
adrenal gland	xxx	xxx	xxx	xxx	xxx

Legend: The number of x indicates the number of animals in which the analyte was detected.

In rodents, T_4 accounts for approximately 50% of the TH secreted by the thyroid gland. Hence, 3-T_1AM could be a major product of T_4 metabolism in the thyroid. Therefore, TH and TAM profiles were analyzed in mouse thyroids. T_4, T_3, 3,5-T_2 and 3-T_1AM were detected in mouse thyroids to be in the range of pmol/g wet weight (Figure 19). While 3,5-T_2 was clearly identified as single peak (Figure 19C), baseline peak separation was not achieved for the two other T_2 iso-

mers, 3,3`-$T_2$ and 3`,5`-T_2, (Figure 19C) observed close to the LOD by the chromatographic method developed herein. 3-T_1AM was measured in the same concentration range as T_4 and T_3 in murine thyroid tissue. The peak intensity for rT_3 was below the LOQ to exactly quantify rT_3 (Figure 19B) in the thyroid gland.

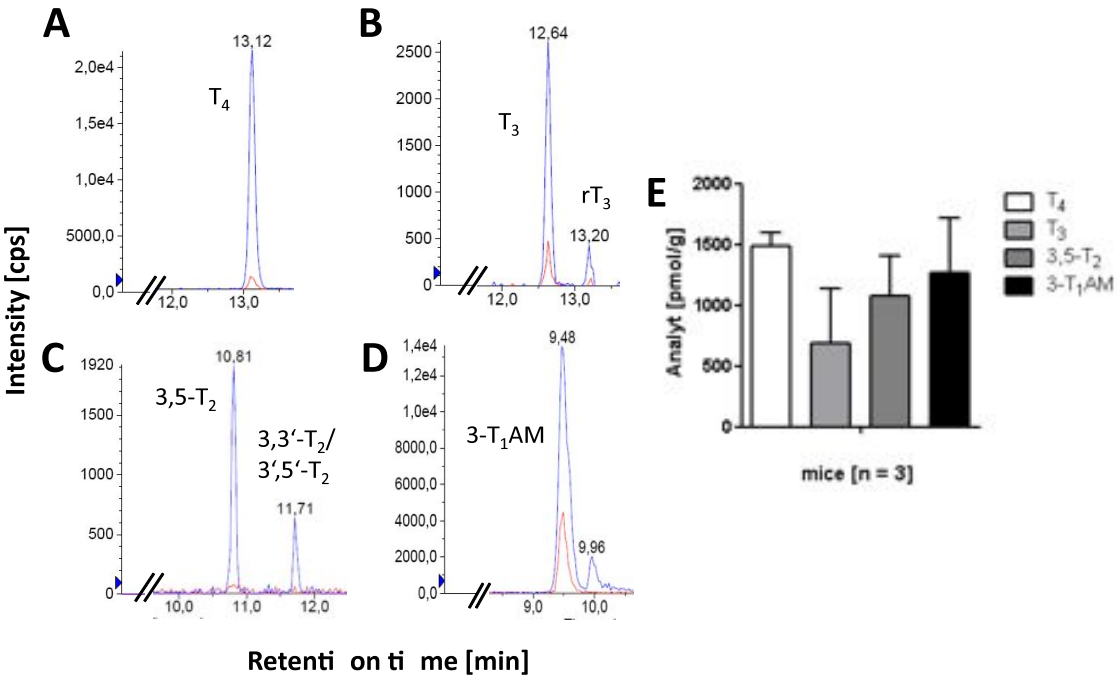


Figure 19: Representative chromatograms (two transitions per compound) (A-D) of the QTRAP®4000 LC-MS/MS analysis and quantification (E) of perfused mouse thyroids.
Endogenous T_4 (A), T_3 (B), 3,5-T_2 (C) and 3-T_1AM (D) were detected above the detection limit. The peak quantification of three perfused mice is shown in Fig. 4 E.

3.1.3 Cellular uptake studies with thyroid hormones

The uptake of TH and 3-T_1AM into cells is a crucial issue to study the cellular action and consequently to understand the physiological role of these messengers. Cellular entry of TH is mediated by plasma membrane transporters which on the other hand do not transport TAM. Hence, cellular uptake of T_3 and 3-T_1AM was also studied using the LLE based LC-MS/MS assay.

3.1.3.1 T_3 uptake in MCT8 transfected MDCK1 cells

In a first step, the LC-MS/MS method was validated for the uptake of T_3 in direct comparison with an established ^{125}I-T_3 uptake assay (Figure 20). The advantage of using the LC-MS/MS is that the uptake of unlabelled ligands into cells can be directly measured.

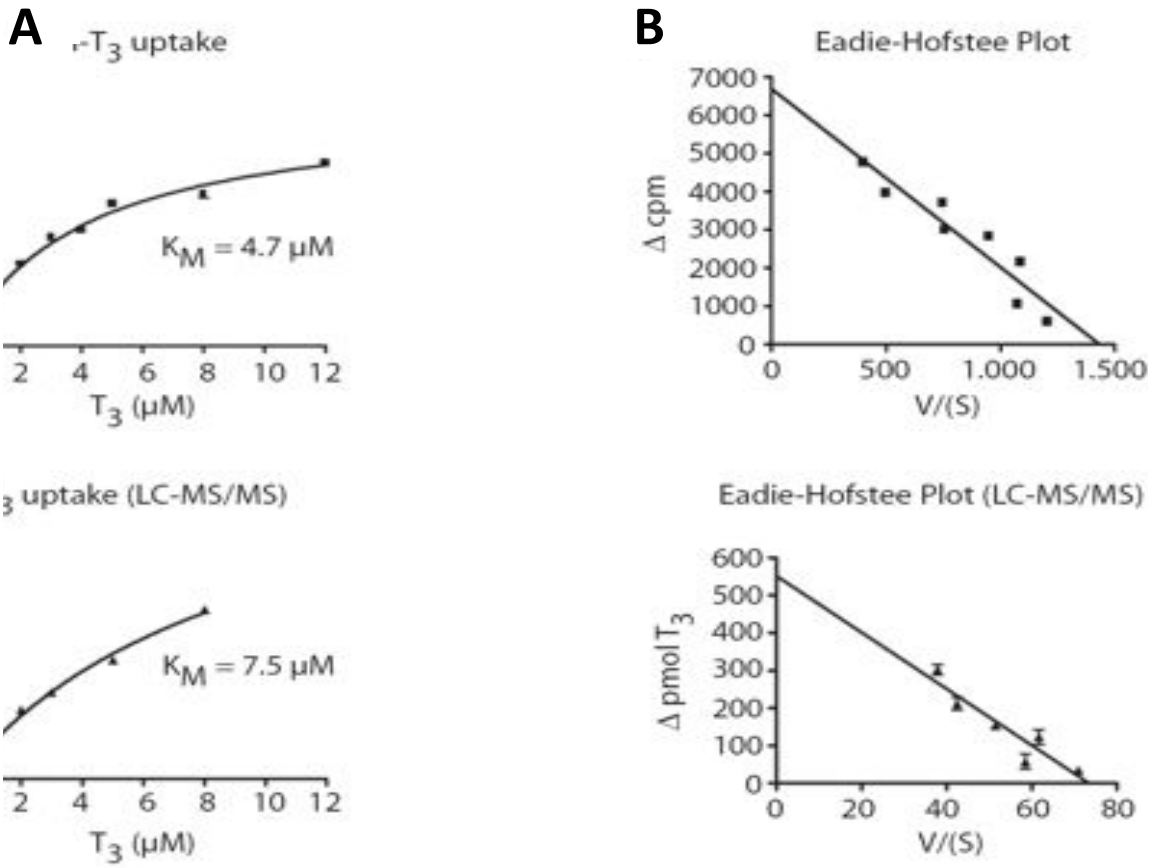


Figure 20: Comparison of T_3 uptake measurements based on ^{125}I-T_3 internalization or QTRAP®4000 LC-MS/MS analysis.

Cell associated radioactivity depending on substrate (T_3) concentration (A). Lines represent fitted binding isotherms. All data points were determined in triplicate and the mean values and standard errors of the mean are indicated. The experiments were performed two times with similar results. Eadie-Hofstee plot based on data from A (B). K_M values were not approximated from the plot, but were directly calculated from the raw data using GraphPad software.

Therefore, MCT8-transfected Madin-Darby canine kidney 1 (MDCK1) cells were incubated for three minutes with T_3 at concentrations ranging from 500 nM to 12 µM containing or not ^{125}I-T_3 as tracer to study the cellular T_3 uptake (the cell culture and radioactive uptake (^{125}I-T_3) part of the experiment was done by Dr. Anita Kinne and published in Kinne, et al., (Kinne, et al., 2010)). Only MCT8 as a known TH transporter transports exclusively TH and does not transport other substrates like amino acids. The K_M values determined by both methods, 4.7 µM by ^{125}I-T3 uptake and 7.5 µM by LC-MS/MS (Figure 20), are within the range reported for rat Mct8, i.e. 4 µM using Xenopus oocytes (Friesema, et al., 2003). Hence, the established LC-MS/MS procedure is comparable with classical uptake methods. Subsequently, the MCT8-mediated uptake of the TH derivatives T_3AM and triac was also investigated in MDCK1 cells using LC-MS/MS. Even at 50 µM, both compounds were not transported by MCT8 (Figure 21) whereas T_3 as a known substrate is transported. Thus, the direct assay shows that MCT8 requires the intact L-amino acid side chain for substrate recognition.

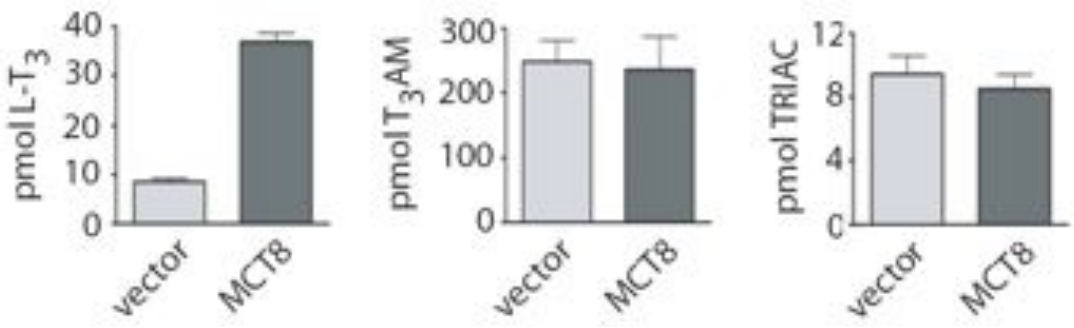


Figure 21: Uptake of T_3, T_3AM and triac by vector and MCT8 transfected MDCK1 cells.
Data are representative of one out of at least two independent uptake experiments performed in triplicate and analysed with the QTRAP®4000 LC-MS/MS. Intracellular T_3, T_3AM, and Triac isolated from the cells after incubation with 10 µM in FCS-containing medium.

3.1.3.2 T_3 uptake in endogenously MCT8 expressing FRTL-5 cells

The rat FRTL-5 cell line is well-studied and widely used in thyroid research. These cells are unable to synthesize and secrete T_3 *in vitro* under monolayer culture conditions. However, many genes involved in TH biosynthesis, cellular uptake, transport (e.g. MCT8) and receptor-mediated signaling of T_3 and 3-T_1AM are expressed in FRTL-5 cells (Figure 22).

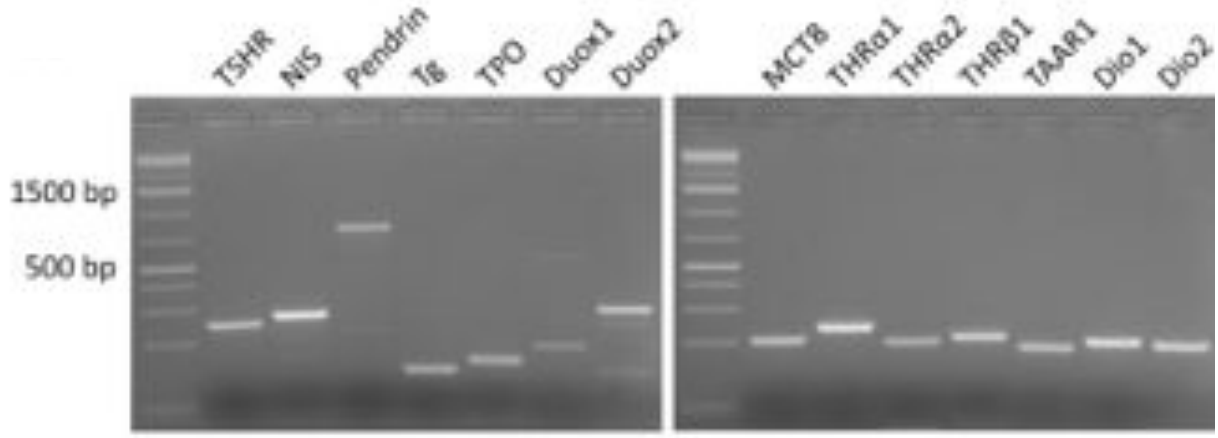


Figure 22: FRTL-5 gene expression concerning TH synthesis, transport and signalling.
A PCR with cDNA from FRTL-5 cells and specific primer was performed. The amount of template cDNA was the same in all samples. This picture shows the result of a gel electrophoresis (1.5 %, TAE-buffer) after the PCR. The GeneRuler™ 1 kb Plus DNA ladder (Fermentas) was used for sizing and approximate quantification of the observed PCR fragments.

To study the TH and TAM uptake in FRTL-5 thyrocytes, which endogenously express MCT8, cells were incubated with 100 nM (200 pmol) T_3 and 3-T_1AM, respectively, for 30 min to study the uptake and metabolism of T_3 and 3-T_1AM. Cells were harvested after 0, 5, 10, 15, 20 and 30 min and cell homogenates were extracted using the LLE and analyzed using the LC-MS/MS (Figure 23). LC-MS/MS analysis of cell lysates after incubation with T_3 or 3-T_1AM showed the successful uptake of the substances into FRTL-5 cells within 30 min. A baseline of T_3 properly resulting from the FCS-containing media at approximately 0.05 pmol was detected in the control experiment with PBS. The intracellular T_3 concentration increased in a linear manner over time, reaching a final level of approximately 0.25 pmol.

In contrast, the kinetics of 3-T_1AM uptake were characterized by an initial, rapid increase of intracellular 3-T_1AM during the first 10 min of incubation followed by a plateau phase at approximately 3 pmol, suggesting saturation, metabolism or export out of the cell (Figure 23).

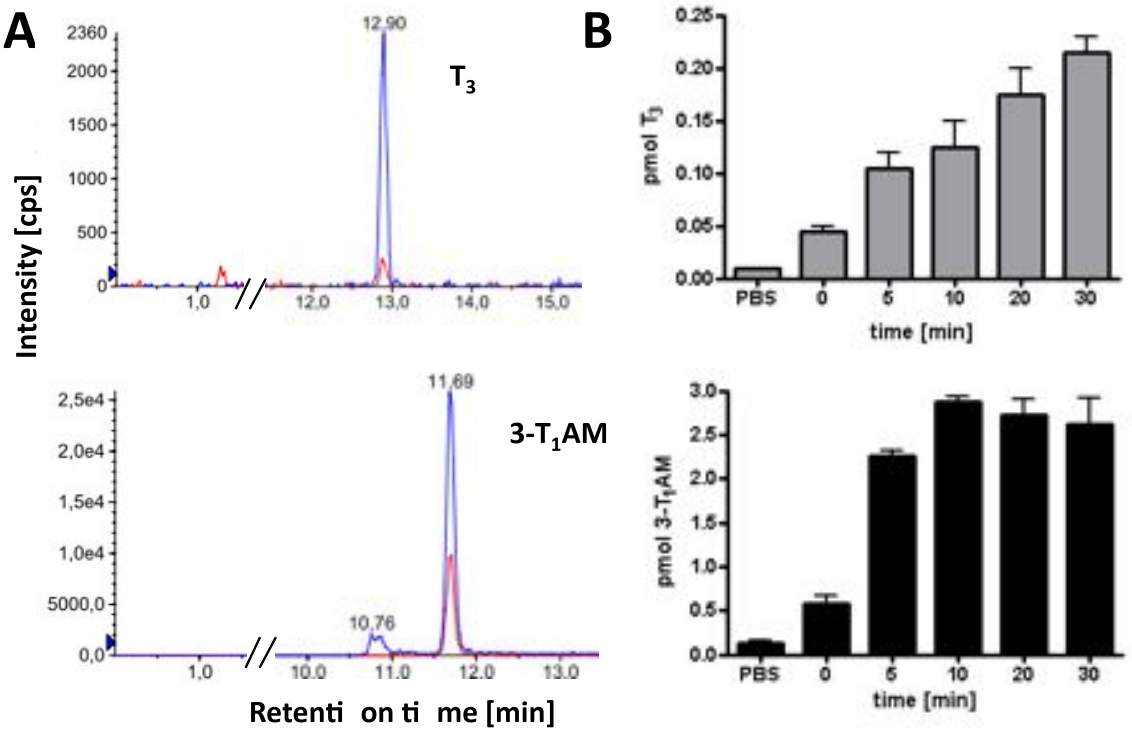


Figure 23: Uptake of T_3 and 3-T_1AM into FRTL-5.

Representative chromatograms (two transitions per compound) of the QTRAP®4000 LC-MS/MS analysis of T_3 (upper panel) and 3-T_1AM (lower panel) after 30 min on FRTL-5 cells. Intracellular T_3 and 3-T_1AM isolated from FRTL-5 cells after incubation with 100 nM (200pmol) T_3 or 3-T_1AM in FCS-containing medium (B).

3.1.4 Solid phase extraction using urea for isolation of TH and TAM

Since the re-discovery of 3-T_1AM in 2004 by Scanlan and co-workers, the extraction of TAM from serum is a great issue (Scanlan, et al., 2004). The first protocol used for the serum extraction was established in the Scanlan laboratory at the Oregon Health & Science University (OHSU, USA) and based on an urea solid phase extraction prior to LC-MS/MS analysis. The protocol was based on a total T_3 extraction procedure using LC-MS/MS published by Tai, et al. (Tai, et al., 2002).

Using the original extraction protocol with our experimental setup using one MS/MS transition per molecule, it was possible to detect endogenous T_0AM, 3-T_1AM, T_3, rT_3 and T_4 in 500 µl human serum (Figure 24). Although the sample volume was relatively high, peaks of 3,5-T_2 and other TH were not detectable and signal intensities for T_4, T_3, and rT_3 were rather low. However, the extraction of these compounds was not reproducible on a daily basis. In most cases, the detection of endogenous 3-T_1AM and T_0AM was not observed, while spiking experiments with 3-T_1AM succeeded. The comparison of the retention times of the endogenous 3-T_1AM with the deuterated IS proved the accuracy of the peak determination.

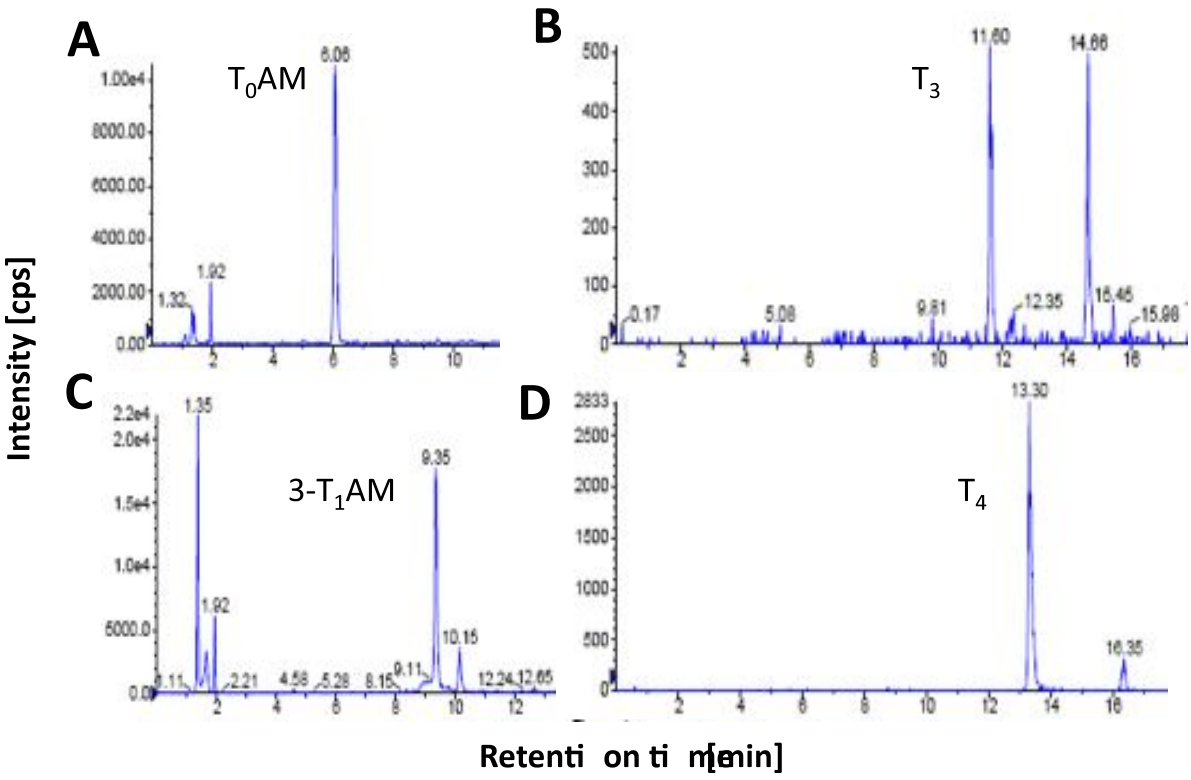


Figure 24: Representative chromatogram of the QTRAP®4000 LC-MS/MS analysis of T_0AM (A), T_3 (B), 3-T_1AM (C), and T_4 (D) in 500 µl human serum.

This chromatogram was recorded before the second transition for each compound was used in the LC-MS/MS method.

All wash fractions of the SPE extraction were collected and analyzed to ensure that 3-T_1AM is not washed out during the preanalytical sample workup. Since this was not the case, several modifications (temperature, reagents, pH, sample volume, sample species, sample matrix, SPE methods) of the protocol were tested and compared to the original protocol (Table 13) to reliably detect 3-T_1AM and other TH (e.g. T_4, T_3, rT_3, 3,5-T_2) in serum.

Table 13 : Variation of the original solid phase extraction protocol.

Parameter	Original protocol	Changes
Incubation temperature	80°C	42°C
Protein binding interfering agent	8M urea	guanidium hydrochloride, protease K
pH range	pH 4.0 -6.0	Variation from acetic to neutral to basic conditions
Protein precipitating agent	Acetone	acetonitrile, phosphoric acid
Sample volume	100 µl	Up to 1,000 µl
Sample species	Human	mouse, rat, Xenopus laevis
Sample matrix	Serum	plasma, urine, saliva
SPE columns	Varian: Bond Elute certify	Sigma-Aldrich: HybridSPE 30mg/ml, HLB-SPE 30 mg/ml
		Waters: Oasis MAX 3cc
		Phenomenex: Strata screen C 150mg/ 3ml, Strata-x-cw 334 60 mg/3ml, Strata-x-c 334 60 mg/3ml

Most of the changes lead to an improved detection of T_4 and T_3, but 3-T_1AM was not detected in any instance. Simultaneous TH and TAM based LC-MS/MS analytics seem to have problems with preanalytical workup and/ or sensitivity of the used LC-MS/MS. After one year of trying to measure the TAM and TH profile of one serum sample in one analytical run, it was decided to focus on separate methods for TH and TAM analysis.

During the SPE optimization process, several SPE columns and procedures were tested. A patent pending SPE technology using 1 ml HybridSPE® cartridge was found which is designed to retain phospholipids. A major cause of ion suppression and matrix effects when using electrospray techniques has been shown to be from endogenous phospholipids. Phospholipid concentrations are present in extremely high concentrations in biological matrices and can vary greatly between subject samples and experimental time points. Using this SPE column, the peak intensities of T_4 and T_3 were above the LOQ using the QTRAP®4000 mass spectrometer (Figure 25 right). However, peak intensities for rT_3 and 3,5-T_2 were mostly below the LOD. To elucidate if the absence of endogenous rT_3 and 3,5-T_2 in serum samples is due to the sensitivity of the LC-MS/MS, the performance of two different tandem mass spectrometers (QTRAP®4000 and 5500 from AB SCIEX) was compared (Figure 25).

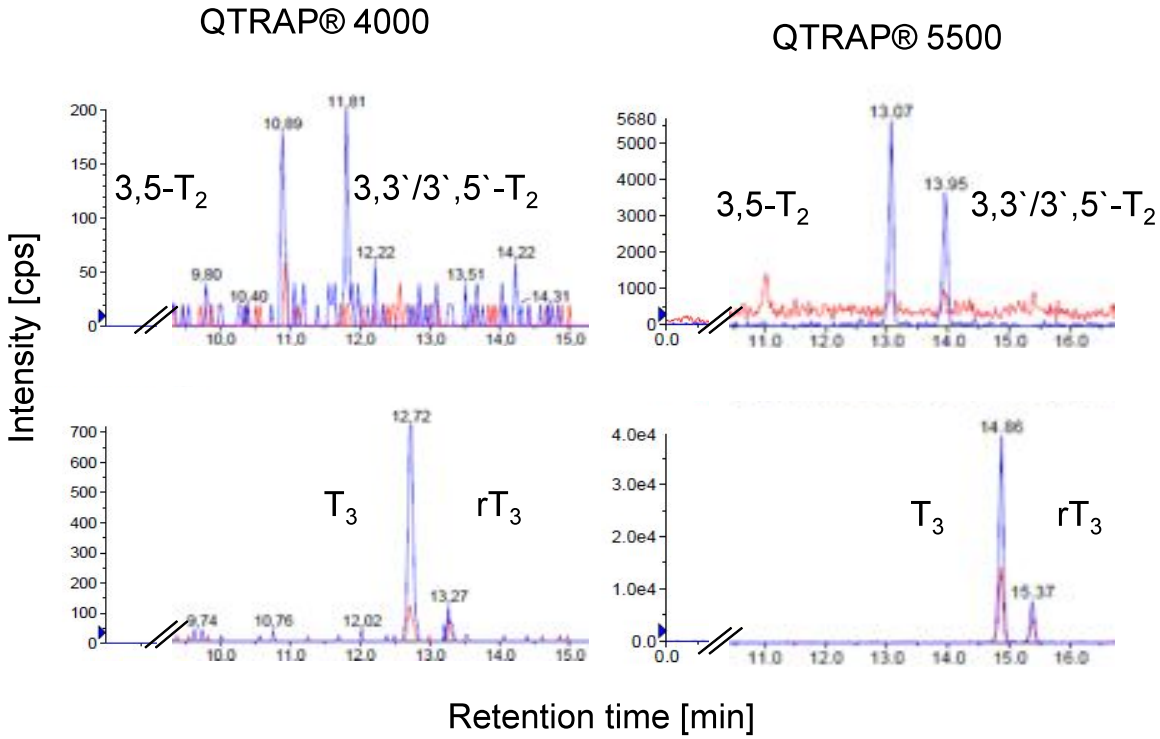


Figure 25: Representative chromatograms (compare different ordinates) of TH analysis using Hybrid SPE of the same human serum analysed with the QTRAP®4000 and with the most sensitive QTRAP®5500 LC-MS/MS system (Prof. Daniel, Molecular Nutrition Unit, TU Munich).

While the QTRAP®4000 only revealed endogenous T_4 and T_3, the more sensitive QTRAP®5500 additionally detects rT_3 and 3,5-T_2 at concentrations allowing quantification in the same human serum which was extracted twice with the HybridSPE® procedure (Figure 25 left). Remarkably, a 4.5-fold increase in peak intensity was achieved in case of lower iodinated compounds like 3,5-T_2 (Figure 26). This is of high relevance as these metabolites also occur at much lower serum concentrations than T_4 and T_3

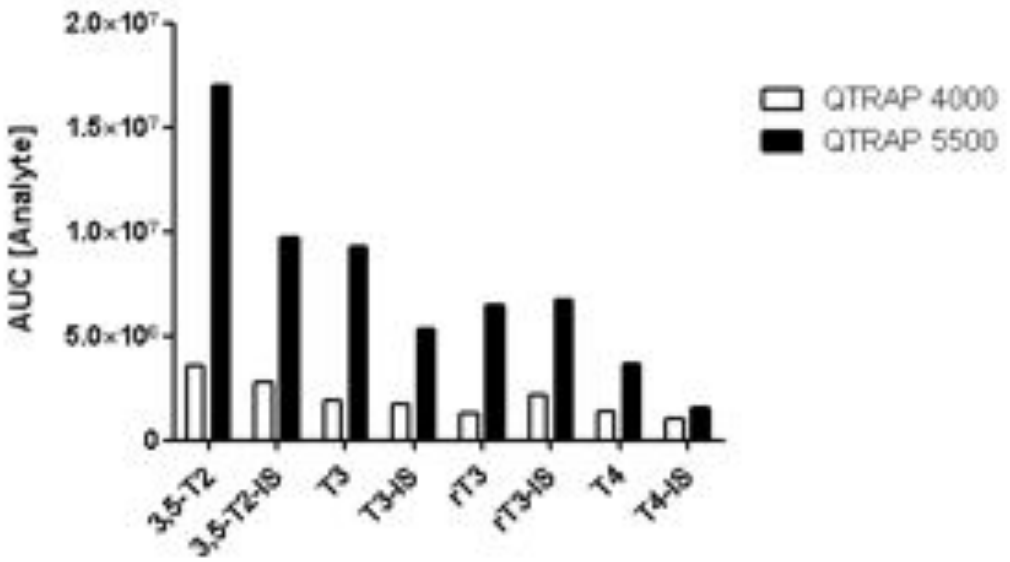


Figure 26: Comparative analysis of signal intensities of 1µM TH standard solution analyzed with the QTRAP®4000 LC-MS/MS system and with the most sensitive QTRAP®5500 system using the identical HPLC separation column and LC chromatographic conditions.

The comparison of the LC-MS/MS devices proofed that the SPE method is worthwhile to analyze several TH, namely T_4, T_3, rT_3, and 3,5-T_2, in 200 µl human serum. However, no endogenous TAM could be detected using the HybridSPE® procedure and the QTRAP®5500 analysis, although spiking experiments with 3-T_1AM for the generation of standard curves worked very well. The complete method validation is still ongoing and is not part of this thesis.

3.2 Development of a chemiluminescent immunoassay targeting 3-T_1AM

Up to now, several SPE based LC-MS/MS methods have been reported for the detection of 3-T_1AM in serum and tissues of various species, but no poly- or monoclonal Ab applicable for construction of a TAM and especially a 3-T_1AM immunoassay had been reported.

3.2.1 Generation of a monoclonal antibody targeting 3-T1AM

MAb targeting 3-T_1AM producing cells were generated using the hybridoma technology first described by Köhler and Milstein (Köhler and Milstein, 1975). It is well understood that the immune response can be stimulated by conjugating small molecules to larger, highly immunogenic carrier proteins. Hence, 3-T_1AM was chemically coupled to bovine serum albumin (BSA; 67 kDa) for successful immunization. Three BALB/c mice were immunized by the injection of BSA-coupled 3-T_1AM to generate MAb targeting 3-T_1AM (Figure 15). After three immunization injections, serum Ab titer assays indicated that all three mice responded with high antibody titers and displayed high antibody avidity for 3-T_1AM-BSA, particularly that from mouse number three. BSA itself as the hapten conjugation partner does not interfere with the assay (Figure 27).

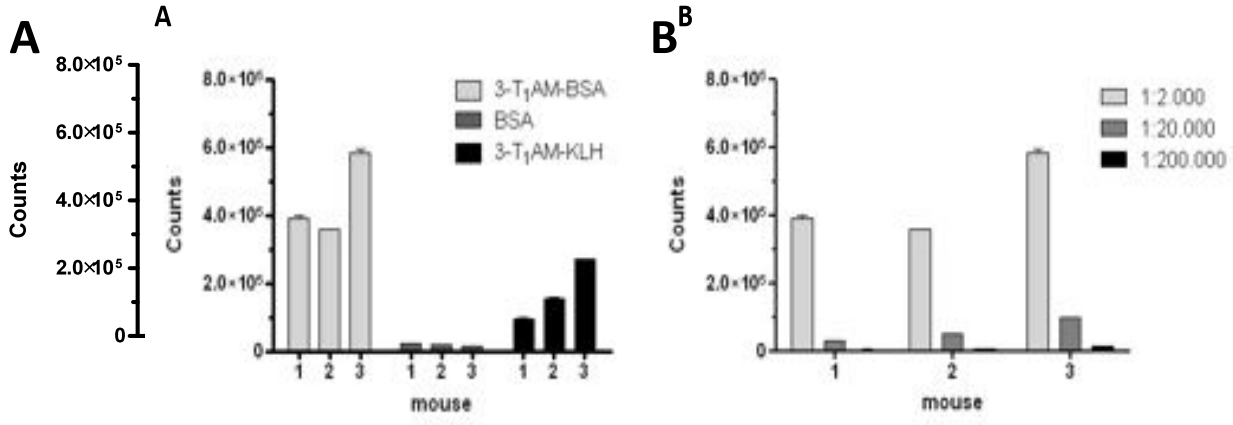


Figure 27: Proof of the specificity of the polyclonal antisera against 3-T_1AM.

Assay results with serum of three mice (A). 3-T_1AM-BSA assay results with different serum dilutions (B). Mouse three showed highest antibody titer with 3-T_1AM coupled to BSA and was used for the production of hybridomas.

More than thirty wells of hybridomas were identified to produce antibodies binding to 3-T_1AM with high affinity. Eight MAb were examined for their cross-reactivity for T_1, T_3 and T_4, and three out of those, termed 5C11, 10D1, and 9C5 with high affinity and specificity to 3-T_1AM were chosen for cloning, expansion and further characterization.

MAb 9C5 showed less than 0.1% cross-reactivity with T_4 and T_3 and less than 1% with most of the other TH, TAM and TAc. Only 3,5-T_2AM and 3-iodo-L-thyroacetic acid (T_1Ac) which so far have not been detected in human serum or tissue by chromatographic or LC-MS/MS methods showed a cross-reactivity of 12,4% and 5,1%, respectively (Table 14). With respect to potential cross-reactivity of Ab for TH, the most important concern is their affinity and detection of the most abundant TH T_4 and T_3. Based on the average total T_4 serum concentration of 100 nM, less than 0.1% cross-reactivity with our antibody to T_4 would lead to a variation of less than 0.1 nM. These data clearly indicate that this particular MAb against 3-T_1AM is highly specific. Normal concentrations of all 3-T_1AM related compounds in serum are given in Table 14. The concentration of each substance is dependent upon the amount synthesized and secreted, their affinity for carrier serum proteins, distribution in tissues, rate of degradation, and clearance.

Table 14: Cross-reactivity and serum concentrations of 3-T_1AM -related molecules.

	Cross-reactivity [%]	Human serum concentration [nM]	reference
Iodothyronamines (TAM)			
T_0AM	< 0.1	n.q.	
3-T_1AM	100	~ 60	(Geraci, 2008), *
3'-T_1AM	< 0.1	n.d.	
3,5-T_2AM	12.4	n.d.	
3,3'-T_2AM	< 0.1	n.d.	
3',5'-T_2AM	< 0.1	n.d.	
T_3AM	< 1	n.d.	
rT_3AM	< 1	n.d.	
T_4AM	< 0.1	n.d.	

	Cross-reactivity [%]	Human serum concentration [nM]	reference
Iodothyronines (TH)			
T_0	< 0.1	n.q.	
3-T_1	< 0.1	0.075	(Corcoran and Eastman, 1983)
3'-T_1	< 0.1	0.059	(Chopra and Sabatino, 2000)
3,5-T_2	< 1	0.055	(Chopra and Sabatino, 2000)
3,3'-T_2	< 1	0.058	(Chopra and Sabatino, 2000)
3',5'-T_2	< 1	0.085	(Chopra and Sabatino, 2000)
T_3	< 0.1	2.1	(Chopra and Sabatino, 2000)
rT_3	< 0.1	0.62	(Chopra and Sabatino, 2000)
T_4	< 0.1	110	(Chopra and Sabatino, 2000)
Iodoacetic acids (TAc)			
Tetrac	< 0.1	7.2	(Chopra and Sabatino, 2000)
Triac	< 0.1	2.8	(Chopra and Sabatino, 2000)
Diac	< 1	n.d.	
T_1Ac	5.3	n.d.	

Legend: n.d., not detected so far in human serum; n.q. not quantified so far in human serum, * this study

3.2.2 Immunoprecipitation of serum using the MAb 9C5

MAb cell clone 9C5 appeared highly specific for 3-T_1AM as demonstrated in Table 14. Additionally, an immunoprecipitation experiment followed by LC-MS/MS analysis was performed with MAb 9C5 to confirm the specificity already seen in the cross-reactivity experiment. The recovery of 100 nM 3-T_1AM added to 7% BSA-PBST as TAM/TH deficient matrix was 82%. Immunoprecipitation with the MAb 9C5 and 5 human serum samples and subsequent analysis of extracted antigens bound to the precipitated MAb 9C5 showed again that the MAb specifically recognizes 3-T_1AM but not T_4 or any other TH or TAM, respectively. A representative chromatogram for d_4-3-T_1AM, 3-T_1AM and T_4 as the TH with the highest concentration in serum is shown in Figure 28.

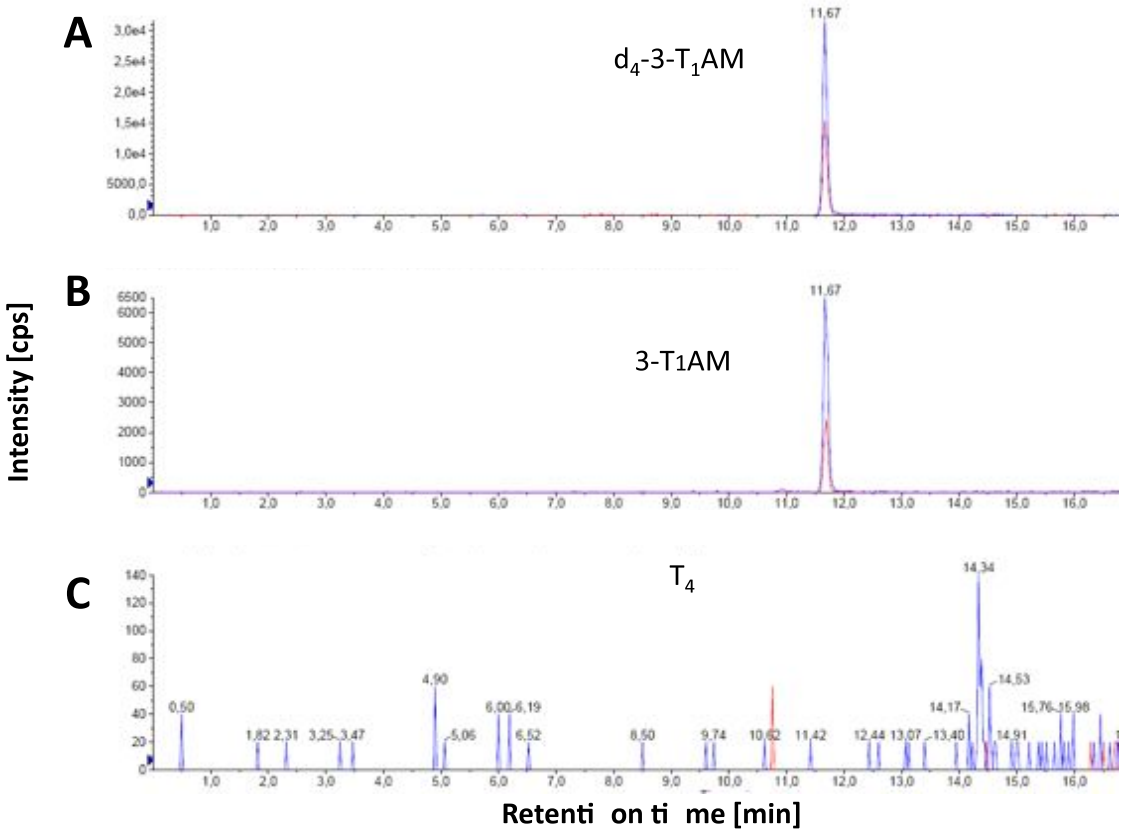


Figure 28: Representative chromatogram of the QTRAP®4000 LC-MS/MS analysis of the extracts bound to the precipitated MAb 9C5 in the immunoprecipitation experiment.

The chromatographic separation and detection of the IS d_4-3-T_1AM (A), 3-T_1AM (B) and T_4 (C) is shown. While the IS and the endogenous 3-T_1AM is detectable, no T_4 is in the extracted sample.

3.2.3 Development of a chemiluminescent immunoassay

MAb 9C5 was selected for construction of the 3-T_1AM chemiluminescent immunoassay (CLIA) due to its high specificity and low cross-reactivity with other TAM.

In the competitive assay procedure, HRP-labeled 3-T_1AM competes with 3-T_1AM in serum for binding to a limited amount of anti-3-T_1AM MAb (Figure 29). In order to obtain the optimal assay conditions, all assay parameters and incubation times were optimized.

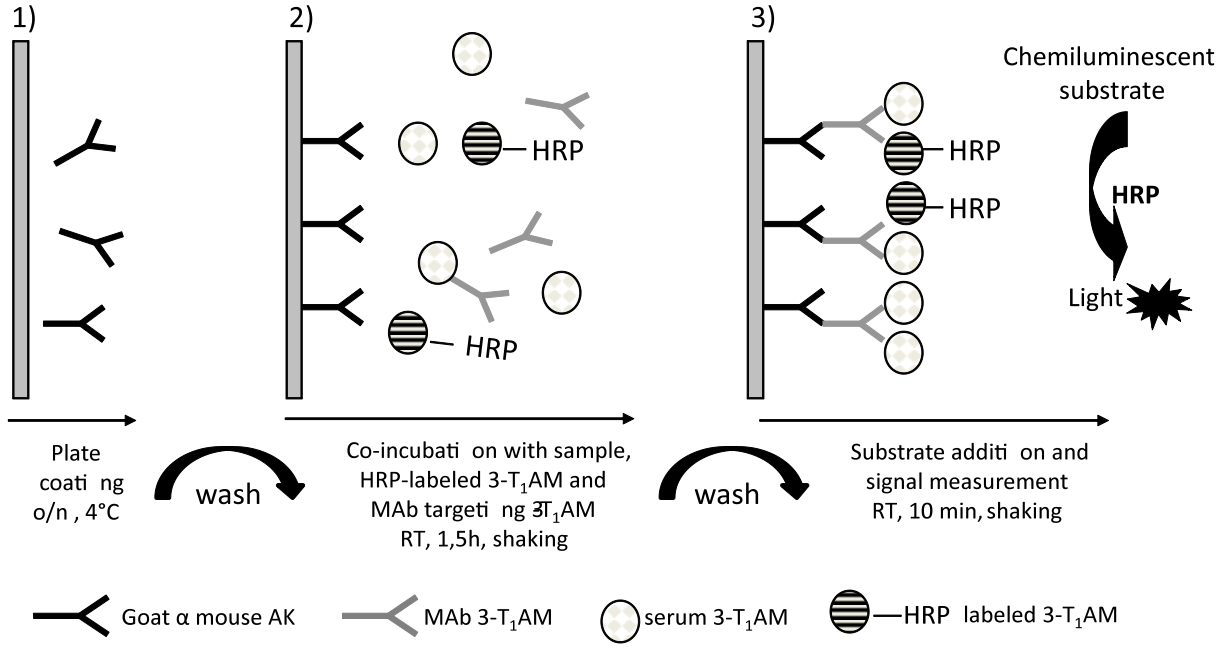


Figure 29: Schematic diagram of the developed 3-T_1AM chemiluminescent immunoassay.

To prepare a standard curve for quantifying 3-T_1AM in human serum, 3-T_1AM deficient human serum is needed. Therefore, several commercially available TH deficient sera were tested using the established immunoassay and 7% BSA-PBST as an artificial, 3-T_1AM deficient serum matrix. Since the types and affinities of serum binding proteins differ between TH and TAM, TH deficient serum can still contain endogenous 3-T_1AM. Four of five tested TH free sera contained 3-T_1AM ranging from 25 to 55 nM 3-T_1AM (Figure 30).

Only the T_3/T_4 deficient serum from Sigma-Aldrich was below the lowest standard and therefore the matrix of choice lacking endogenous 3-T_1AM. This serum is delipidated with fumed silica and dextran-coated charcoal treated to remove steroid hormones. Consequently, this T_3/T_4 and TAM deficient serum was used to construct the assay standard curve ranging from 0 to 500 nM 3-T_1AM. A typical standard curve using T_3/T_4 deficient serum or 7% BSA –PBST for the present assay is shown in Figure 31.

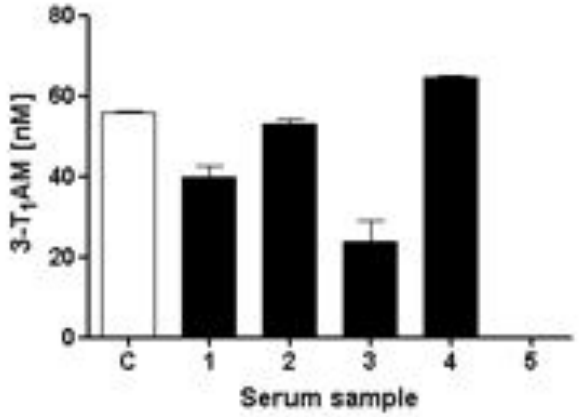


Figure 30: 3-T_1AM analysis of five different T_3/T_4 deficient sera compared to normal control.

Lyphocheck serum (Bio-Rad, München, Germany) served as positive control. Serum 1+ 2: two different charges of human, defibrinated, T_3/T_4- free serum (PAN BIOTECH GmbH, Aidenbach, Germany). Serum 3: TSH, T_3 & T_4 depleted serum and 4: T_3/T_4 depleted processed serum (SCIPAC, Sittingbourne, United Kingdom). Serum 5: T_3/T_4 deficient serum (Sigma-Aldrich, Taufkirchen, Germany).

The ratio of counts/ $counts_{max}$ was used for calculation of the standard curve. Both standard curves are highly parallel suggesting comparable matrix effects. The linear concentration range of the 3-T_1AM assay is from 10 to 200 nM.

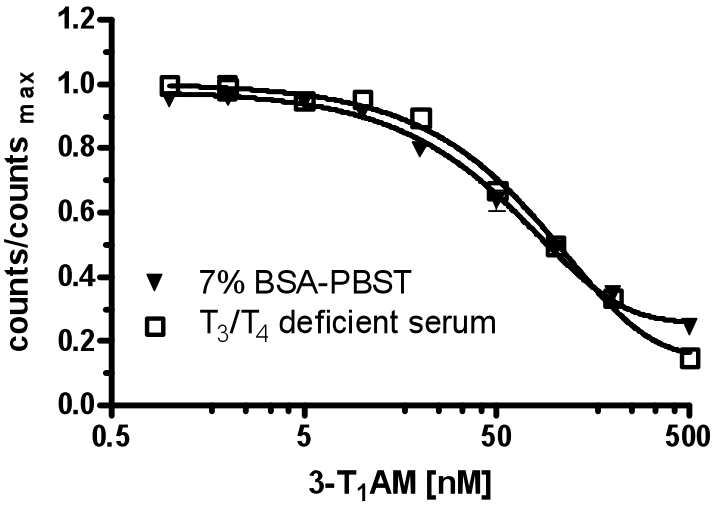


Figure 31: Standard curves for 3-T_1AM using the CLIA.

Increasing concentrations of non-labelled 3-T_1AM were added to 7% BSA-PBST (▼) or T_3/T_4 deficient serum (□) to displace binding of the 3-T_1AM-HRP tracer.

3.2.4 Validation of the immunoassay

Dilution linearity was assessed by assaying 3-T_1AM standard samples after serial dilution with T_3/T_4-deficient serum. Measured concentrations were multiplied by the dilution factor and compared with the original concentration. The results show a good agreement between the expected and the observed values in the steep part of the standard curve (Figure 31). The working range of the assay is estimated between 10-500 nM. The inter-assay precision of the assay was 18.5% and the intra-assay precision was 10.8%.

Recovery of 3-T_1AM measurement was tested by comparing the data of 5 sera in different combinations of equal volume mixtures of two sera each (Figure 32 left). Measured concentrations from each mixture of two sera were added up in order to obtain the theoretical value for the measurement of the equal volume mixtures. Results agreed with the theoretically expected arithmetic means for the corresponding samples and was <5% for all mixed samples.

Furthermore, recovery of 3-T_1AM spiked to serum in rising concentrations (0-50 nM) was studied (Figure 32 right). Mean recovery of the theoretical values to the measured data determined by the assay was < 5% for all spiked samples.

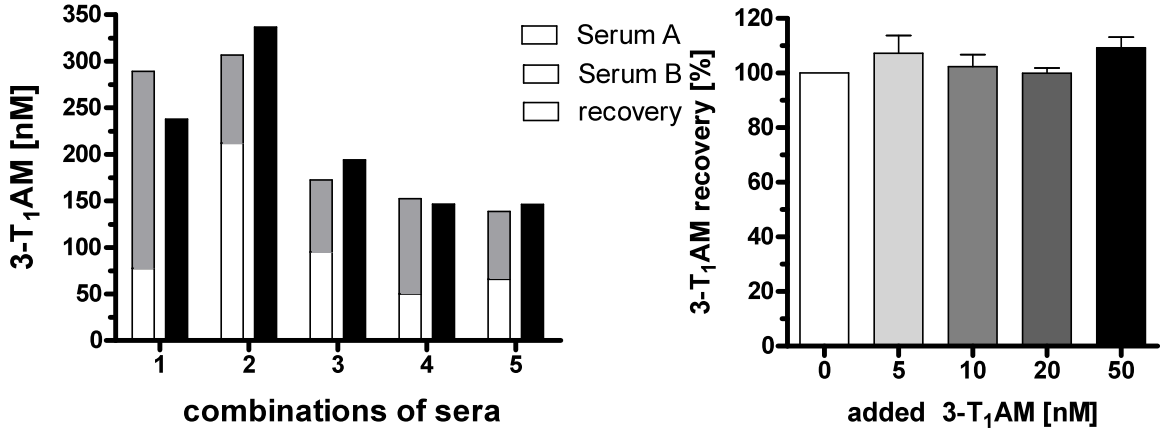


Figure 32: 3-T_1AM immunoassay validation.

In this recovery study, different combinations of mixtures of two sera were measured, results were summed up (two-columns, left) and are presented along with the calculated concentration (grey column, right) (left). The recovery of 3-T_1AM spiked into serum in rising concentrations (right).

The stability of 3-T_1AM was also tested in serum. 3-T_1AM was stable at RT and 4°C overnight (83% and 93%, respectively) as well as after four freeze-thaw-cycles [98%] (Figure 33).

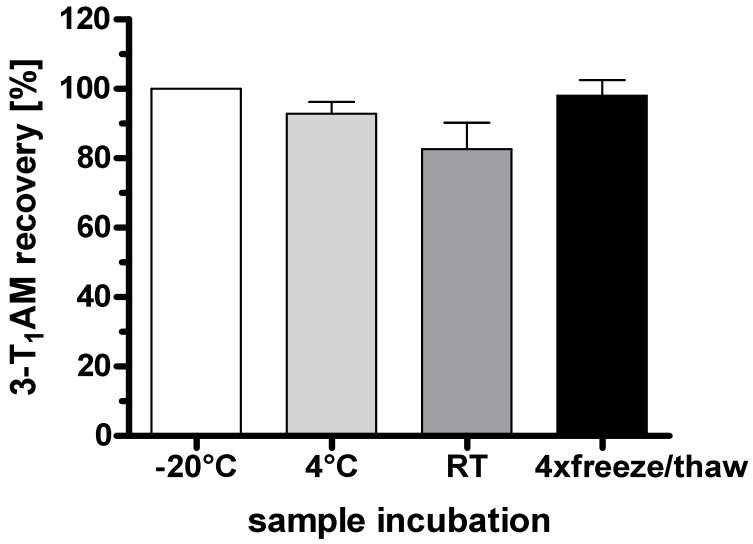


Figure 33: Stability of 3-T_1AM in human serum.

Stability of 3-T_1AM in serum stored at RT or 4°C overnight and after 4 freeze/thaw cycles.

In healthy human serum, 3-T_1AM was reliably detected and quantified in the nM concentration range by the CLIA. Human plasma and serum are widely used matrices in clinical and biological studies. However, different collecting procedures and the coagulation cascade influence concentrations of proteins and metabolites in these matrices (Yu, et al., 2011). Therefore, the 3-T_1AM concentrations in EDTA plasma and serum samples collected simultaneously from ten individuals were analyzed. No statistical difference, but a slight trend towards lower 3-T_1AM levels in plasma was observed (Figure 34).

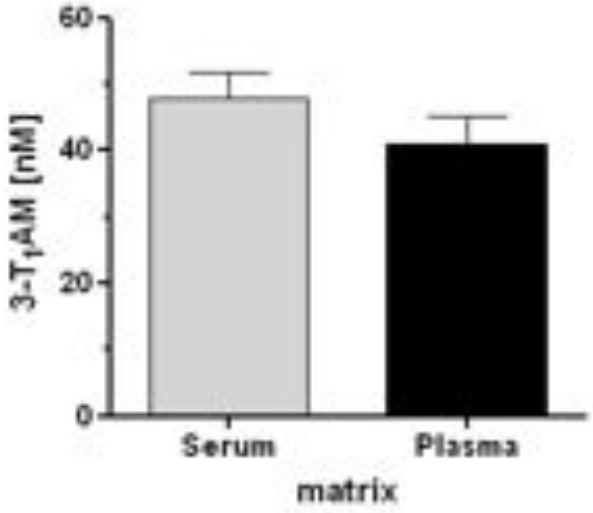


Figure 34: 3-T_1AM concentration of sera and plasma of 10 healthy individuals.
There was no statistical difference (unpaired t-test).

3.2.5 3-T_1AM binding studies

Agents, like 8-anilino-1-naphtalene sulfonic acid and sodium salicylate, which interfere with the TH binding proteins are often used for total TH measurements. Sodium salicylate was shown to interfere with T_3 and T_4 binding to TBG (Christensen, 1959; Larsen, 1972). In contrast to TH, a high fraction of 3-T_1AM (> 90%) is specifically bound to apoB100, but not to the classical TH binding proteins. Therefore, the influence of binding proteins to 3-T_1AM concentrations was studied analysing the role of free vs. total 3-T_1AM. In the first description of serum analysis from DeBarbar, et al. (DeBarber, et al., 2008), 8 M urea at 80°C for 30 min was used to dissociate 3-T_1AM from its binding protein (Figure 35 left). Addition of 8 M urea to Lyphocheck, a commercially available human standard serum, increases 3-T_1AM concentration, but as illustrated with NaCl as reference material, urea itself increase 3-T_1AM in the CLIA to the same extent as in Lyphocheck.

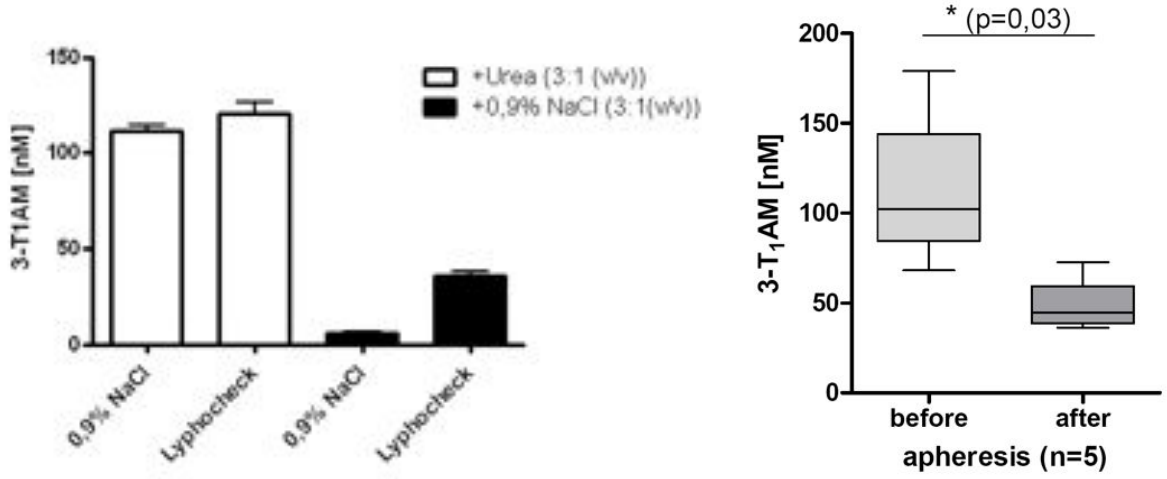


Figure 35: 3-T_1AM binding studies.

3-T_1AM binding studies using 8 M urea as agent interfere with binding proteins (left). NaCl served as negative control and diluting agent (n=2, measurement in duplicate). 3-T_1AM serum concentration in five patients before and after lipoprotein apheresis (right).

The therapy of lipoprotein apheresis is used for patients with hereditary familial hypercholesterolemia showing additional risk factors for cardiovascular diseases (Thompson, 2010). Since lipoprotein apheresis is being performed with increasing frequency and 3-T_1AM binds tightly to apoB100, 3-T_1AM concentration before and after lipoprotein apheresis in 5 patients was investigated. Before lipoprotein apheresis the mean 3-T_1AM serum concentration was 111 ± 40 nM and significantly decreased to 48 ± 14 nM after therapy (Figure 35 right).

3.2.6 3-T_1AM is mainly produced extrathyroidally

The median serum concentration of 3-T_1AM in 13 adult healthy subjects was 66 ± 26 nM and therefore comparable with previous LC-MS/MS results (Geraci, 2008) and in the same nM concentration range as the thyroid pro-hormone T_4. In sera of 105 thyroid cancer patients on TSH suppressive L-T_4 therapy 3-T_1AM levels were significantly higher than in controls (Figure 36).

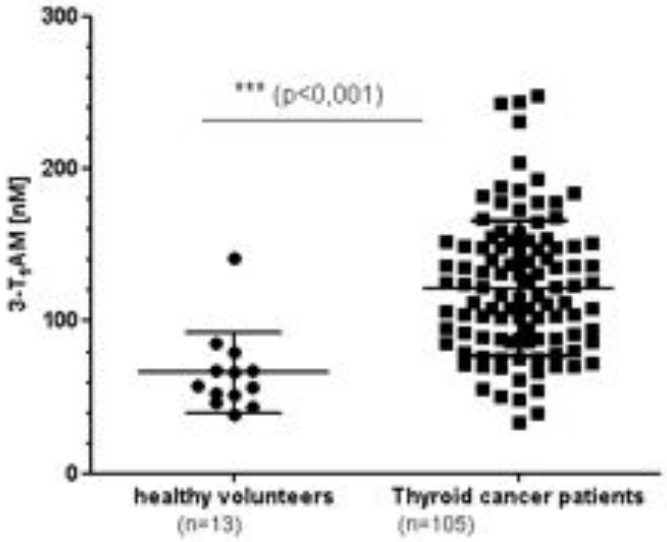


Figure 36: 3-T_1AM levels in 13 healthy individuals and 105 thyroid cancer patients.

Median values are indicated by horizontal lines. There was a significant difference in median values between the two groups (unpaired t-test, (*** p<0,001)).

However, there was no significant correlation between 3-T_1AM levels and TSH (Figure 37 left) or fT_4 (Figure 37 right), respectively, in the thyroid cancer cohort. In the whole group of thyroid cancer patients 3-T_1AM was age-independent, but a trend towards slightly higher levels in sera of females (125 nM, n=80) than in those of males (110 nM , n=25) was observed while not reaching statistical significance (Figure 38).

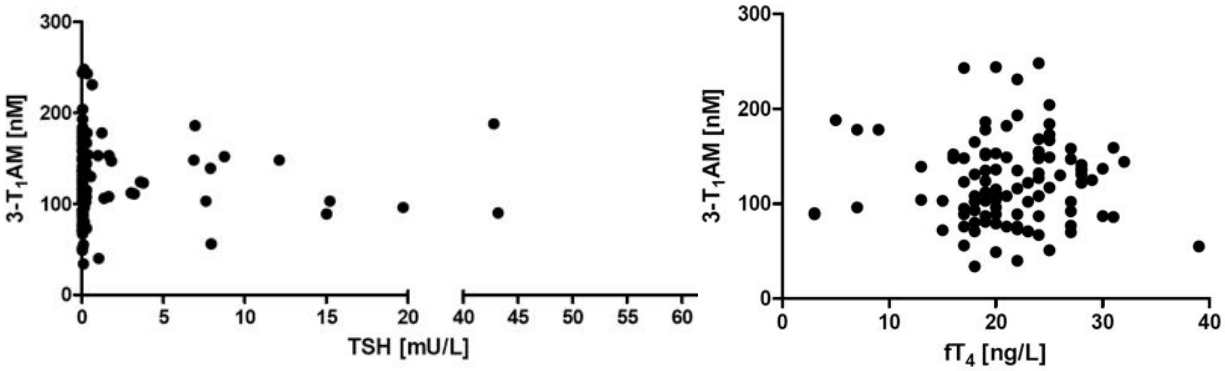


Figure 37: Correlation of 3-T_1AM and TSH (left) in the thyroid cancer cohort.

There was no correlation or statistical significance.

Figure 38: Correlation of 3-T_1AM and age (male patients left, female patients middle) and sex (right) in the thyroid cancer cohort.

There was no correlation or statistical significance.

3-T_1AM was also detected in 10 T_4-substituted patients with pituitary insufficiency. While fT_4 [18.1 vs. 11.4 nM] and fT3 [4.9 vs. 3.7 nM; p< 0.001 in both] significantly decreased during T_4 withdrawal after 6 days in these patients, 3-T_1AM levels remained constant [97 vs. 92 nM], suggesting differences in homeostatic regulation between these TH metabolites (Figure 39).

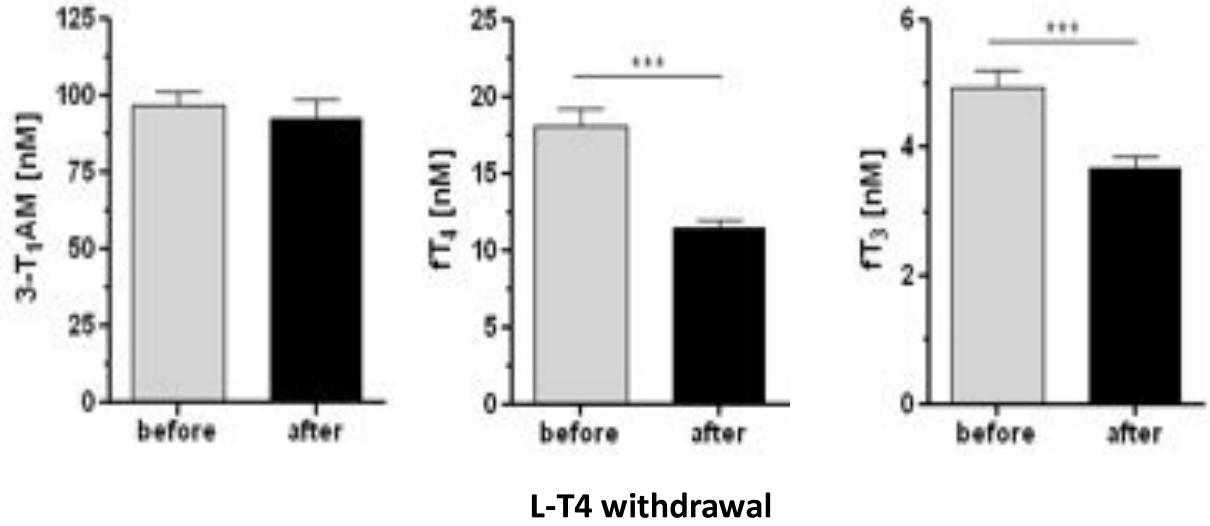


Figure 39: Exploratory study of 10 T_4-substituted patients with pituitary insufficiency before and after 6 days of L-T_4 withdrawal.

Serum concentrations of 3-T_1AM (left). fT_4 (middle) and fT_3 (right) in this cohort. Statistical significance was calculated using paired t-test (*** p<0,001).

3.2.7 3-T_1AM levels in murine serum

Since the established human 3-T_1AM assay was developed with a goat-anti-mouse Ab as catching Ab, the coating protocol was modified for the measurement of murine serum. To reliable measure murine 3-T_1AM, the MAb 9C5 was directly coated onto the plate (Figure 40).

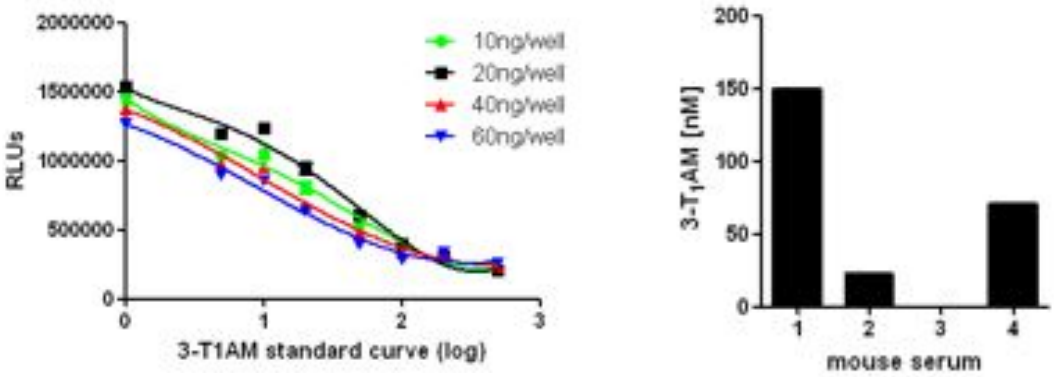


Figure 40: 3-T_1AM standard curves of four different coated plates using different MAb concentrations (left) and four different mouse serum with 40 ng/well coated plates (right).

Different MAb concentrations were tested using 3-T_1AM standard curves diluted in 7%-BSA-PBST (Figure 40 left). Finally, 40 ng/well MAb was chosen to measure 3-T_1AM in different mouse sera (Figure 40 right). In one mouse serum (number 3), no 3-T_1AM could be detected creating a 3-T_1AM deficient serum for the generation of standard curves. However, in all other sera 3-T_1AM levels ranged from 23 to 150 nM.

3.3 The role of the AADC in thyronamine biosynthesis

TAM exhibit great structural similarity to TH, and biosynthesis from TH appears likely. The biosynthetic pathway would require decarboxylation and one or more deiodination steps to convert e.g. T_4 into a 3-T_1AM. It was shown previously that TAM are substrates for DIO and several possible pathways were suggested, but the decarboxylating enzyme which was postulated remained elusive. The PLP-dependent enzyme AADC has been suggested several times as the TH decarboxylating enzyme (Braulke, et al., 2008; Doyle, et al., 2007; Scanlan, et al., 2004).

3.3.1 Verification of AADC enzymatic activity using L-DOPA

To test the hypothesis that AADC converts TH into TAM, decarboxylation of L-DOPA to dopamine served as a positive control. Using the QTRAP®4000 LC-MS/MS, the detection of L-DOPA and dopamine as well as almost all added TH and TAM standards was possible. This enabled us to study the decarboxylation reaction. In order to confirm that the recombinant human AADC preparation was active and functioning properly, the enzyme was incubated with one of its known substrates, L-DOPA.

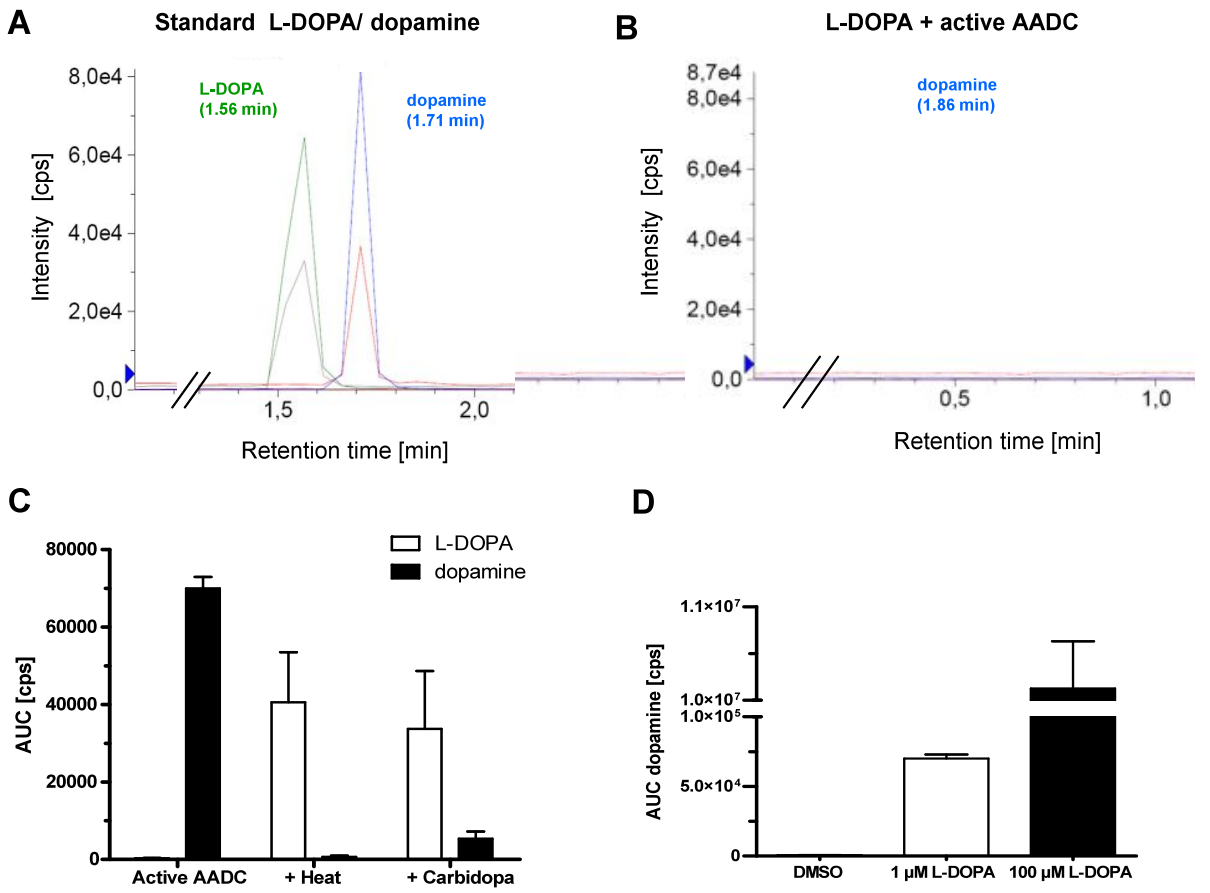


Figure 41: The decarboxylation of L-DOPA to dopamine by AADC as positive control.

LC-MS/MS detection (two transitions per compound) of standard solutions of L-DOPA and dopamine. (A). QTRAP®4000 LC-MS/MS detection (two transitions) of dopamine after incubation of AADC with 1 µM L-DOPA (conditions: 20 min, 37°C, pH 6.8; n=3) (B). The decarboxylation of 1 µM L-DOPA to dopamine by AADC is inhibited by heat and carbidopa (n=3) (C) and concentration-dependent (conditions: 30 min, 37°C, pH 6.8; n=3) (D). Data show the area under the curve (AUC) in counts per second (cps).

Figure 41A shows the chromatographic separation of a standard solution of L-DOPA and dopamine. In Figure 41B the result of the 30 min incubation of L-DOPA with the AADC is shown. Incubated L-DOPA was quantitatively converted into dopamine. The decarboxylation to dopamine was inhibited both by heat-inactivation and by the specific AADC inhibitor Carbidopa (Figure 41C). Under the reaction conditions chosen, dopamine formation increased in a concentration-dependent manner (Figure 41D). These results show that the AADC preparation used for all experiments was enzymatically active on its proper substrate L-DOPA.

3.3.2 AADC does not decarboxylate thyroid hormones

In order to test whether the active AADC preparation also decarboxylates TH, the experiment from Figure 41B was repeated under standard incubation conditions but with TH as substrates.

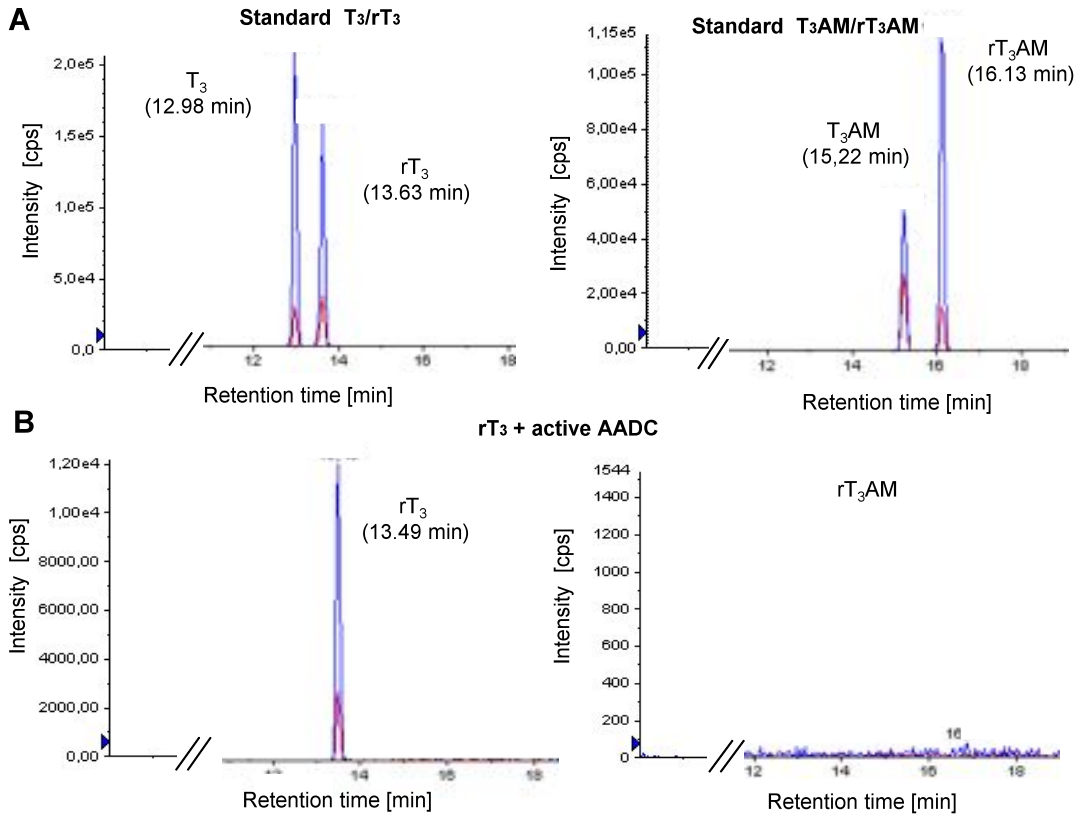


Figure 42: AADC does not catalyse the conversion of rT_3 to rT_3AM.

QTRAP®4000 LC-MS/MS analysis (two transitions per compound) of T_3, rT_3, T_3AM and rT_3AM standard solutions (A). LC-MS/MS analysis two transitions) of the incubation of 100 nM rT_3 with active AADC (conditions: 30 min, 37°C, pH 6.8, n=3) (B).

Figure 42A shows the chromatographic separation of a standard solution of T_3, rT_3, T_3AM and rT_3AM. Figure 42B shows a representative result of the incubation (30 min) of 100 nM rT_3 with active AADC. There was no conversion of rT_3 to rT_3AM. This experiment was performed with all other eight possible TH (T_4, T_3, 3,5-T_2, 3,3'-T_2, 3',5'-T_2, 3-T_1, 3'-T_1, T_0) without detecting any TAM formation (not shown). Higher concentrations of TH (up to 5 µM) were also assayed to rule out the possibility of weak binding due to a high Km, but no TAM formation was observed. To check further if the possible precursor of TAM is 3-T_1 or 3,5-T_2 instead of rT_3, we repeated this experiment and also increased the incubation time up to 3 h. Neither a 3-T_1AM (Figure 43A) nor a 3,5-T_2AM (Figure 43B) peak was detected but the substrates 3-T_1 and 3,5-T_2 were still found in the incubated sample.

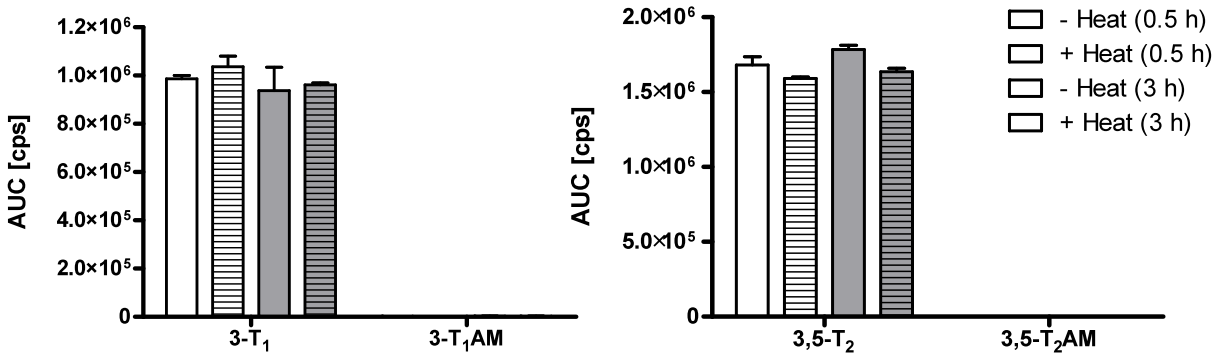


Figure 43: Incubation of 3-T_1 and 3,5-T_2 as potential precursors of TAM.

LC-MS/MS analysis of the incubation of 3-T_1 (A) and 3,5-T_2 (B) with AADC (conditions: 37°C, pH 6.8, 30 min and 3 h, respectively). Heat-inactivated AADC served as negative control.

3.3.3 Influence of incubation temperature, time and pH on AADC activity

Incubation conditions, namely pH (Figure 44A), temperature (Figure 44B), and reaction time (Figure 44C), were varied to test whether this might enable the decarboxylation of rT_3 to rT_3AM. Figure 44A-C show the area under the curve of rT_3 and rT_3AM at different pH levels, temperatures and time-points. However, rT_3AM was not detected in any of the samples, showing that AADC cannot catalyze the decarboxylation of rT_3 to rT_3AM.

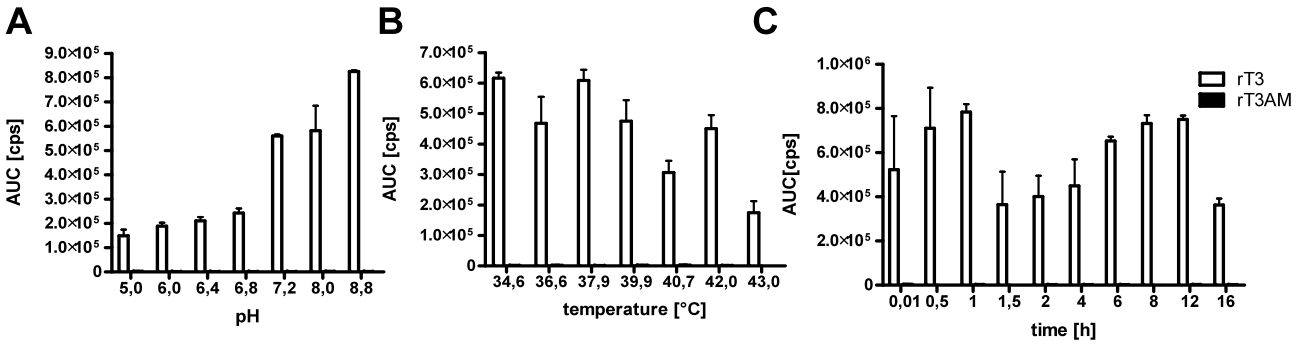


Figure 44: Influence of pH, temperature and time on the incubation of rT_3 with AADC.

QTRAP®4000 LC-MS/MS analysis of the incubation of rT_3 with AADC. Influence of pH: conditions: 30 min, 37°C, pH 5.0-8.8, n=3 (A). Influence of temperature: conditions: 30 min, 34.6°C - 43.0°C, pH 6.8, n=3 (B). Influence of time: conditions: 1 min-18 h, 37°C, pH 6.8, n=3 (C).

3.3.4 3-T_1AM concentration in patients with *AADC* deficiency

If AADC was involved in TAM biosynthesis, one would expect lower levels in patients deficient in *AADC*. 3-T_1AM concentrations in plasma samples of four anonymous patients with biochemically confirmed *AADC* deficiency were measured using the newly established 3-T_1AM CLIA. The mean plasma concentration of 3-T_1AM was 46 ± 18 nM and thus comparable (unpaired Mann Whitney test, p= 0.2339) with healthy control (n=13, 66 ± 26 nM) (Figure 45). Consequently, this supports the obtained *in vitro* findings that AADC is not involved in TAM biosynthesis.

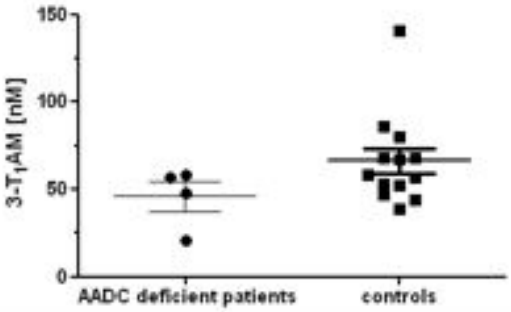


Figure 45: 3-T_1AM plasma concentrations in four patients with biochemically confirmed *AADC* deficiency and 13 healthy controls.

Mean values are indicated by horizontal lines. There was no statistical difference between both groups (unpaired Mann Whitney test, p= 0.2339).

4 Discussion

TAM represent a novel class of TH-derived endogenous signalling compounds with remarkable biological effects on core body temperature, cardiac function, energy metabolism, and neurotransmission (reviewed in Piehl and Hoefig, et al. (Piehl, et al., 2011)). 3-T_1AM is the major TAM found in serum and tissue, but its physiological concentration measured with LC-MS/MS or other methods is still under discussion. TAM are formed from their precursor TH via a combination of deiodination and decarboxylation in target tissues (Saba, et al., 2010). T_4AM and its derivatives with lower iodine content are substrates of the three DIO enzymes (Piehl, et al., 2008). However, the putative decarboxylase proposed to generate TAM from their corresponding TH is still elusive.

Hence, the aims of the thesis were the establishment of the TH and TAM analysis in different matrices using LC-MS/MS as well as the identification of the TH decarboxylase(s) necessary to generate 3-T_1AM *in vivo*.

4.1 Analytics of 3-T_1AM and thyroid hormone metabolites

One essential objective of this thesis was the analysis of all possible TH and TAM metabolites in different matrices of a single sample and in one analytical LC-MS/MS run. During the past years, sensitive LC-MS/MS based methods were the best choice to study endocrine parameters like steroids, vitamin D or TH in a single sample and in different matrices (Adamec, et al., 2011; Surowiec, et al., 2011; Thibeault, et al., 2012; Vogeser and Parhofer, 2007).

LC-MS/MS is a technique that identifies the analyte by the mass-to-charge ratio of parent and fragmentation ions and the HPLC retention time. The quantification of analytes is mainly achieved by determining the ratio of analyte to isotopically labeled internal standard, a method called isotope dilution. LC-MS/MS assays are highly accurate and have low limits of detection, and most importantly they are specific for the analyte (Jonklaas, et al., 2009; Vogeser and Seger, 2012).

4.1.1 LC-MS/MS measurement of 3-T_1AM and thyroid hormone metabolites

Several LC–MS/MS methods have been developed to measure free (non-protein bound) and total (free + protein bound) TH and their metabolites in human serum or plasma (Gu, et al., 2007; Kunisue, et al., 2010; Lembcke, et al., 2005; Soldin and Soldin, 2011; Soldin, et al., 2005; Yue, et al., 2008). The diagnostic accuracy of total TH measurements would only be proportional to that of free TH if all individuals would have similar binding protein concentrations. In consensus with the free hormone hypothesis, only the free fraction of TH is responsible for biologic effects (Bartalena, et al., 1996; Ekins, 1992; Mendel, et al., 1988). Hence, the measurement of free TH concentrations in serum has been accepted to be clinically useful and the more physiological parameter, especially in case of serum binding-protein abnormalities like pregnancy (Bartalena, et al., 1996; Bhatkar, et al., 2004; Kahric-Janicic, et al., 2007).

In 2004, Scanlan, et al. presented for the first time the detection of 3-T_1AM and T_0AM in rodent serum and tissues using LC-MS/MS. It was astonishing that the extraction and detection of 3-T_1AM mainly in serum would be so challenging. Especially the identification of its high affinity serum binding protein apoB100 highlights the analytical differences between TH and TAM (Roy, et al., 2012). While TH and TAM are highly similar in terms of structure, they prefer different serum binding and transport proteins, in serum, plasma or blood and also receptors. This discrepancy is also underlined by the fact that procedures employed to generate commercially processed TH deficient sera of different companies do not automatically eliminate 3-T_1AM as shown in this project (Figure 30). Although the detection of free and total TH in serum using LC-MS/MS is increasingly used in research and even in clinical laboratory medicine, controversies still exist about the physiological 3-T_1AM serum concentration using LC-MS/MS (Ackermans, et al., 2010; Saba, et al., 2010; Soldin, 2009). All attempts so far to extract endogenous 3-T_1AM from human serum and to efficiently and quantitatively recover 3-T_1AM using LC-MS/MS gave inconsistent results. Reproducible protocols may only provide the free or at least a fraction of non-apoB100 bound 3-T_1AM. Using the API®5500 LC-MS/MS as the currently most sensitive LC-MS/MS on the market, Soldin, et al. published a LC-MS/MS method for the detection of free 3-T_1AM in serum (Soldin, 2009).

Concerning human serum, the focus in this project was on the determination of TAM (T_0AM, 3-T_1AM) and TH metabolites (T_4, T_3, rT_3, 3,5-T_2). In cooperation with Prof. Dr. Daniel (Molecular Nutrition Unit, TU Munich), the parallel measurement of human serum samples with

the QTRAP®5500 LC-MS/MS and with our QTRAP®4000 (both from AB SCIEX) was done. Whereas the QTRAP®4000 only detects T_4 and T_3 in serum, the more sensitive QTRAP®5500 additionally detects the TH metabolites rT_3 and 3,5-T_2 above the LOQ with safe signal intensities over noise. However, endogenous 3-T_1AM was still not detected using this sensitive LC-MS/MS system suggesting good SPE extraction efficiencies for TH but not for TAM. Access to a QTRAP®5500 LC-MS/MS would provide the opportunity to test different SPE and LLE methods for the reproducible detection of 3-T_1AM. The ambitious goal to measure all TH and TAM in one sample was not yet achieved in serum as a suggested matrix during this project. Especially in case of serum analytics, the chemistry and biochemical properties (e.g. serum binding proteins) of TH and TAM differ too much to extract both substance classes by one common extraction procedure.

4.1.2 Detection of endogenous 3-T_1AM and TH metabolites in tissue samples

Several publications using LC-MS/MS or immunological methods already reported tissue TH concentrations in the pmol/g concentration range (Ackermans, et al., 2012; Kunisue, et al., 2010; Obregon, et al., 1981; Saba, et al., 2010). In contrast, to the difficulties of TAM detection using serum, excellent measurement of 3-T_1AM is obtained in samples from *in vitro* studies, cell culture homogenates and tissue extraction samples. In these matrices, 3-T_1AM is not bound to serum apoB100 which is complicating the extraction procedure.

Geraci, et al. found high (pmol/g wet weight) 3-T_1AM tissue concentrations in normal human thyroid tissues, suggesting 3-T_1AM may be synthesized by the thyroid gland, and in tissues previously shown to be affected by exogenous 3-T_1AM administration or accumulating 3-T_1AM such as skeletal muscle and adipose tissues (Geraci, 2008). Saba, et al. observed that the endogenous 3-T_1AM was higher in each analyzed rat organ (i.e. liver, kidney, muscle, heart, lung, and brain) than in rat blood suggesting a tissue specific production and/or accumulation of 3-T_1AM (Saba, et al., 2010). However, the difference between “high” tissue and “low” serum levels of 3-T_1AM (Saba, et al., 2010) could be due to extraction problems of 3-T_1AM in serum. It is not mentioned, if the samples were of euthyroid rats and if the animals were perfused in order to remove or minimize residual blood containing TH bound by serum transport proteins TBG, TTR and albumin. Nevertheless, it seems that 3-T_1AM acts like a real endocrine hormone which is secreted by a cell or a gland directly into the bloodstream.

In many of the analysed perfused mouse organs in this project, T_4, T_3, rT_3, 3,5-T_2, and 3-T_1AM were detectable suggesting a wide tissue distribution of endogenous 3-T_1AM. In mouse thyroids, 3-T_1AM was measured in the same concentration range (pmol/g wet weight) as T_4 and T_3. Up to now, it is not clear if 3-T_1AM is only taken up from the blood by the highly vascularized thyroid or if 3-T_1AM is produced from TH within the thyroid tissue. However, endogenous 3-T_1AM thyroid content is still higher compared to other tissues. Studying the effect of Tg proteolysis and decarboxylase inhibitors on thyroidal TH and TAM concentration as well as mouse thyroids with different genetic TH axis modifications will help to understand the physiological role of 3-T_1AM in the thyroid and to understand the possible 3-T_1AM-based regulation of TH biosynthesis.

The uptake of exogenous radiolabeled 3-T_1AM from the blood stream revealed that 3-T_1AM is taken up by all tested organs (Chiellini, et al., 2012). A significant increase in tissue vs. blood concentration occurred in gallbladder, stomach, intestine, liver and kidney reflecting a biliary excretion and enterohepatic recycling of 3-T_1AM. These radiotracer studies reveal that liver, muscle and adipose tissues should be regarded as 3-T_1AM storage sites.

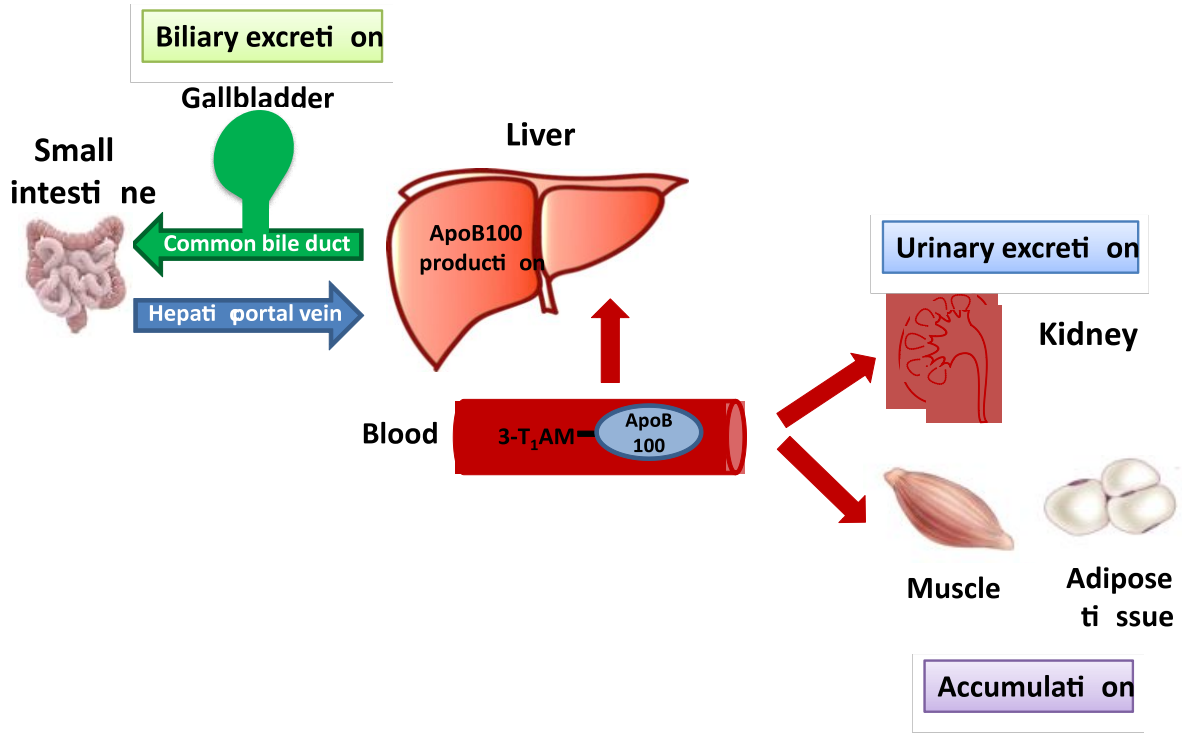


Figure 46: Tissue distribution, biliary excretion and enteric reabsorption of 3-T_1AM.

4.1.3 Cellular uptake of 3-T_1AM and T_3 in FRTL-5 cells

The known molecular targets of 3-T_1AM include both intracellular (e.g. DIO, Sulfotransferase, MAO) and plasma membrane proteins (e.g. TAAR1), suggesting that cellular transport of 3-T_1AM may be important for its biological action (Ianculescu, et al., 2009). Other structurally related compounds, including TH and biogenic amines are translocated across plasma membranes by various cellular transporters (Kristensen, et al., 2011; Visser, et al., 2011). 3-T_1AM has recently been found to be transported into a variety of cell types via a specific transport mechanism which can be saturated and inhibited (Ianculescu, et al., 2009). No single specific TAM transporter has been identified up to now; however, the sodium and chloride independent, pH dependent, TAM specific intracellular transport may involve multiple transporters (Ianculescu, et al., 2009). 3-T_1AM is not transported into the cell via TH transporters (Ianculescu, et al., 2010; Ianculescu, et al., 2009; Kinne, et al., 2010; Scanlan, et al., 2004). 3-T_1AM itself inhibits TH transporters like MCT8 or MCT10 (Ianculescu, et al., 2010).

The time-dependent uptake of 3-T_1AM and T_3 by the differentiated thyroid follicular cell line FRTL-5 which is derived from normal rat thyroid could be demonstrated in this work. In particular in case of T_3, its successful uptake into the cell is a prerequisite for biological action, since the THR is localized in the nucleus. T_3 can be transported into the cells by TH transporters like MCT8 expressed in FRTL-5 cells. Due to the localization of the 3-T_1AM receptor TAAR1 or other potential GPCR targets in the cell membrane, its transport into the cells is not essential for the induction of biological effects. However, 3-T_1AM uptake into the cells would increase the number of feasible signaling pathways. The cellular FRTL-5 uptake of 3-T_1AM and TAAR1 expression in FRTL-5 cells and human thyroid tissue was independently shown by Agretti, et al. (Agretti, et al., 2011). FRTL-5 cells are able to take up, accumulate, deiodinate and oxidatively deaminate exogenous 3-T_1AM providing evidence for uptake into the cell (Agretti, et al., 2011).

Roy, et al. postulated that 3-T_1AM can be taken up via the LDL-receptor (Roy, et al., 2012). The LDL-receptor is a cell surface receptor that mediates the uptake of LDL particles from the bloodstream via receptor-mediated endocytosis (Hobbs, et al., 1990). FRTL-5 cells possess high affinity LDL receptors which bind, internalize, and degrade LDL (Bifulco, et al., 1990). Hence, the cellular uptake of 3-T_1AM into FRTL-5 cells via endocytosis is also feasible but not proven until now.

4.1.4 Immunoassays to measure thyroid hormones and their metabolites

Currently, serum-based immunoassays are available for measuring both total TH (T_4, T_3, and rT_3) concentrations as well as the free TH (T_4 and T_3). However, the difficulties with immunoassays to measure accurately TH that circulate in low concentrations in the bloodstream are well-known and some are mentioned below. Especially free TH immunoassays are known to be susceptible to fault. Jonklaas, et al. postulated that LC-MS/MS methods are more specific than immunoassays for TH measurement and quantification (Jonklaas, et al., 2009). TH autoantibodies (THAA) disrupt the equilibrium between TH and their binding proteins. This may lead to false estimations of free T_4 and free T_3 measurements (Vyas and Wilkin, 1994). A multicentre comparison of free TH immunoassays showed that a large variability in measured values is still present (Giovannini, et al., 2011). Hence, the reference value for free TH is individual for each assay type (direct or indirect measurements). Beside the assay type, the free TH value is influenced by the assay temperature. A temperature study by Soldin, et al showed that both free T_4 and free T_3 concentrations are 1.5 times higher at 37°C than at 25°C (Soldin, et al., 2005). Remarkably, several studies have shown that the binding affinity of TBG for TH is very sensitive to temperature variations (Ross and Benraad, 1992; van der Sluijs Veer, et al., 1992). Other endogenous substances like heterophilic antibodies, lipids, immunoglobulins, indoxyl sulphate, indole acetic acid and hippuric acid in serum may interfere with TH measurements when immunoassays are used (Ghosh, et al., 2008; Iitaka, et al., 1998; Soldin and Soldin, 2011). The blood of pregnant woman contains a lot of non-specific heterophilic antibodies, and these clearly affect the validity of total and free TH measurements. In non-pregnant women, there is a fairly good agreement between immunological and LC-MS/MS methods for total T_4. But during pregnancy, the correlation between immunoassay and LC-MS/MS results became poorer and the correlation coefficients in fact decreased with increased gestational age (Kahric-Janicic, et al., 2007). Effects like this need to be validated for the newly established 3-T_1AM immunoassay.

4.1.5 Detecting 3-T_1AM in serum using the newly established immunoassay

Besides the rapid development of LC-MS/MS based methods for a wide number of biochemical analytes, classical MAb-based immunoassays represent the predominant tool used for routine analysis of endocrine parameters. At the beginning of this thesis, no poly- or monoclonal antibody to establish a sensitive 3-T_1AM immunoassay in serum had been developed. As it has been thought to be very difficult to raise antibodies against 3-T_1AM for

development of an immunoassay, the detection of 3-T_1AM using LC-MS/MS after extraction with deuterated 3-T_1AM was used for quantitative analysis. The development of a highly specific MAb against 3-T_1AM distinguishing this metabolite from numerous other structural analogs like other TAM, TH and TAc seemed to be unlikely. Most of these compounds only differ in the number and/ or position of an iodine atom or the deaminated/ decarboxylated side chain. No significant cross-reactivity or interference between 3-T_1AM and the tested analogues was observed. However, cross-reaction may still exist between the antibody and other, yet untested TH analogues like TH/TAM sulfates or glucuronides.

There were some publications describing the generation of MAb against small molecules like T_4 and dopamine (Chagnaud, et al., 1987; Doi, et al., 1998). On the basis of the generation of a MAb against T_4 using EDC as a coupling reagent and BSA as a carrier protein, the 3-T_1AM-BSA hapten was formed via the amino group of the side chain of 3-T_1AM. This type of coupling was daring because the side chain is the structural unit where TAM differ from TH and TAc. Using the hybridoma technique, MAb were produced to develop a highly specific MAb-based quantitative CLIA for quantification of 3-T_1AM in human serum. The observed nM concentration range of endogenous 3-T_1AM in human serum provide additional evidence that this re-discovered TH metabolite is of high physiological relevance. Gender-specific differences as observed in the thyroid cancer cohort can be due to sex-differences in apoB100 serum levels (Matthan, et al., 2008). The major advantage of this immunoassay is the elimination of an extraction step. Thus, the higher serum levels measured with this technique in comparison to LC-MS/MS likely reflect more efficient capture of total serum 3-T_1AM than methods involving treatment of serum with denaturing organic solvents which may change chemical 3-T_1AM properties. As often observed in immunoassays, the 3-T_1AM concentrations differ between plasma and serum samples. However, many factors as mentioned above interfere with immunological methods. More studies are necessary to clarify which factors disturb the newly 3-T_1AM assay. Nevertheless, the immunoassay retains the advantages of low cost, low sample volume requirement, robustness, simplicity, high throughput, and adequate sensitivity. If the assay will also be applicable to measure serum of other species remains to be seen. Clinicians are mostly interested in obtaining serum-free T_4 concentrations for the diagnosis of thyroid disease (Soldin and Soldin, 2011). The “free hormone” concept is generally accepted as an appropriate measure for T4 but more clinical studies are required to investigate whether this will also be true for 3-T_1AM in the context of the avid physiological binding of 3-T_1AM to apoB100.

4.1.6 Explaining the differences of 3-T_1AM levels measured with the 3-T_1AM CLIA and LC-MS/MS methods

Using the newly established CLIA, human 3-T_1AM serum concentrations are found in the same nM concentration range as the thyroid pro-hormone T_4, whereas most studies using LC-MS/MS measured 3-T_1AM levels in the pM range. However, the CLIA was not the first method, detecting such high 3-T_1AM concentrations in human serum. In 2008 Geraci, et al. showed at the ATA meeting 2008 in Chicago (USA) the first endogenous 3-T_1AM nM concentrations in human tissues and serum using a SPE extraction and LC-MS/MS method.

One reason for the analytical discrepancy between both methods might therefore be the pre-analytical sample preparation in LC-MS/MS studies which require protein precipitation, SPE/ LLE or sample adsorption to the required precolumn of the LC system. Most LC-MS/MS procedures need such a step for sample concentration on the LC precolumn and/or protein/lipid removal before sample application, whereas the established immunoassay works with native serum samples requiring no pretreatment. All available LC-MS/MS methods involve an extraction step for 3-T_1AM and it is not known yet which extraction methods are needed to efficiently extract endogenous 3-T_1AM that is by all measures tightly bound to serum proteins. Adding synthetic 3-T_1AM IS to serum and using these to gauge extraction efficiency does not accurately replicate the extraction efficiency of endogenous 3-T_1AM.

A high fraction of 3-T_1AM might be precipitated during the sample workup. If the total 3-T_1AM serum concentration is in the nM concentration range and the circulating apoB100 concentration range from 1.5 - 3.0 µM, then the 3-T_1AM serum binding sites are in excess compared to the total 3-T_1AM serum concentration suggesting that it is largely bound- and not free- in circulation (Roy, et al., 2012). Additionally to the studies by Saba, et al. (Saba, et al., 2010) and Soldin, et al. (Soldin, 2009), the estimation of 3-T_1AM in serum would indicate a free fraction of only 1‰ detected by LC-MS/MS while the CLIA taking advantage of a high affinity and avidity MAb might be able to efficiently compete with apoB100 and thus quantify total 3-T_1AM. The free fraction of 3-T_1AM in serum might be as low as that of T_4, which is bound to the high affinity binding proteins TBG and TTR in addition to albumin. All of these TH binding proteins prefer alanine [TH] and acetic acid [TAc] side chain derivatives but not decarboxylated side chain derivatives [TAM]. Maybe the amino-group of TAM is protected by apoB100. This property may distinguish 3-T_1AM from other biogenic amines with short half-lives in circulation for which no such high affinity binding proteins have been reported.

In conclusion, LC-MS/MS based methods require preanalytical SPE/LLE extraction of human serum. This step might not (yet) be working perfectly at recovering 3-T_1AM and yield results something closer to free non-protein/lipid-bound 3-T_1AM or only a fraction of total/bound 3-T_1AM. The finding that circulating 3-T_1AM is largely bound to apoB100 may explain the discrepancy in the reported serum levels of 3-T_1AM using different bioanalytical methods (Roy, et al., 2012). The CLIA might effectively compete for apoB100-bound 3-T_1AM thus allowing a better approximation of total (including (lipo-)protein bound) 3-T_1AM. For the future, determination of apoB100 in samples and correlation with 3-T_1AM will help to understand the physiological consequence of the 3-T_1AM binding to a lipoprotein.

The advantages and disadvantages of both methods detecting endogenous 3-T_1AM in human serum are summarized in Table 15.

Table 15: Advantages and disadvantages of two methods detecting endogenous 3-T_1AM

Parameter	SPE-based LC-MS/MS	3-T_1AM CLIA
Species	Independent	Challenging for rodents (MAb)
Sample volume	200-1,000 µl	25 µl
Preanalytical sample preparation	SPE serum preparation	None
Sample number	Single column extraction	96-Well Plate (40 samples)
Assay time	2 days / 40 samples	½ day / 40 samples
Costs	100 €/40 samples	~ 30 € /40 samples
Specificity	high	MAb dependent
Sensitivity	high	MAb dependent
Interference factors	Matrix (ion suppression and/or ion enhancement), recovery	e.g. Free fatty acids, lipids, heterophilic antibodies
Human serum concentration	~ 0.3 nM	~ 60 nM
Free vs. total 3-T_1AM	Free (+ bound 3-T_1AM ?)	Free + bound 3-T_1AM (=total)

4.1.7 The serum analysis of 3-T_1AM reminds on the measurement of ghrelin

The inconsistent analysis of 3-T_1AM reminds endocrinologists e.g on the measurement of ghrelin, an acetyled gastrointestinal peptide. Ghrelin is involved in the regulation of energy balance due to its effects on the stimulation of food intake and weight gain. The mature 28-amino acid peptide is cleaved from its precursor preproghrelin and is characterized by a very

peculiar structural modification, since the hydroxyl group of serine in position 3 is covalently acylated by an n-octanoic acid residue. This chemical modification is necessary for ghrelin to bind to the ghrelin receptor and to exert biologic activity (Gualillo, et al., 2003). Both acetylated and desacetylated ghrelin forms are present in blood and both forms can be measured using different assays (Yoshimoto, et al., 2002). In the bloodstream, triglyceride-rich lipoproteins mostly transport octanoylated ghrelin whereas high-density lipoproteins and very high-density lipoproteins transport both octanoylated and desacetylated (De Vriese, et al., 2007). Nevertheless, the latter form is the dominant form (~ 90%) in the circulation but the inactive one (Yoshimoto, et al., 2002). Since the discovery of ghrelin at the end of 1999, most studies describe the use of serum whereas only a few studies used plasma. Gröschl, et al. investigated the preanalytical influences on the measurement of ghrelin (Groschl, et al., 2002) using a commercially available RIA. They found out, that ghrelin was stable at 4°C for up to three days, whereas storage at 25 °C for >1 day produced significantly lower results. Repeated freezing and thawing had no influence on the ghrelin concentrations while the ghrelin concentration is lower in citrate and lithium-heparinate plasma than in the serum samples (Groschl, et al., 2002). Moreover, other factors like the body weight influence the ghrelin measurement. Ghrelin levels in the plasma of obese individuals are lower than those in leaner individuals (Cummings, et al., 2002). And anorectic patients have high plasma ghrelin levels compared to both the constitutionally thin and normal-weight controls (Germain, et al., 2007). Hence, the detailed description of patient samples analyzed in a study is necessary to classify the measured value. Furthermore, hormones like ghrelin are secreted in a circadian manner influencing the absolute value (Cummings, et al., 2002). Beside plasma and serum, several groups used already saliva to measure ghrelin levels and suggest an association of ghrelin level in saliva and serum (Aydin, 2007; Aydin, et al., 2005; Groschl, et al., 2005; Li, et al., 2011).

4.2 Biosynthesis of thyronamines

TAM are novel endogenous signalling molecules whose biosynthetic pathway is still to be discovered. It is important to understand the biosynthesis of this natural iodine-containing compound. Recent developments in the analytics of the extraordinary thyroid metabolite, 3-T_1AM, together with several TH metabolites could help to answer this question.

Due to their great structural similarity to TH, biosynthesis of TAM from TH is conceivable. 3-T_1AM and T_0AM can theoretically be derived from TH by removal of the carboxylate group on the amino acid backbone in addition to deiodination, which could occur either before or after decarboxylation. Deiodination of various TAM by the three DIO enzymes has been demonstrated *in vitro* in human cell lines (Piehl, et al., 2008). However, the TH decarboxylase, the sequence of reactions and locations of biosynthesis is still elusive at the moment (Wu, et al., 2005). If 3-T_1AM is produced by a combination of deiodination and decarboxylation of TH, 3-T_1AM could be one of the major end-products of TH metabolism.

In vitro and *in vivo* experiments have not been able to answer the biosynthesis question until now. On the one hand, slow but measurable 3-T_1AM production has been shown from T_3 in rat H9c2 cardiomyocytes providing for the first time evidence of TH as TAM precursor. On the other hand, Ackermans, et al. e.g. could not provide evidence for TAM biosynthesis *in vivo*. They could not detect any endogenous 3-T_1AM or T_0AM in rat serum, human plasma or thyroid tissue using an online SPE extraction combined with LC-MS/MS analysis (Ackermans, et al., 2010). The reason for this observation could be due to an insufficient limit of detection and/ or preanalytical issues such as protein binding (e.g. apoB100) or rapid degradation of TAM by amine oxidases (MAO and others). In addition, they treated rats for ten days with $^{13}C_6$-T_4 in different doses expecting to see the biotransformation to $^{13}C_6$-T_1AM. Although, they detected $^{13}C_6$-T_4 and deiodinated $^{13}C_6$-T_3 in plasma and tissue samples, they did not determine deiodinated and decarboxylated $^{13}C_6$-T_1AM. Since they detected 3-T_1AM and T_0AM only after exogenous administration, they raised questions on the biosynthesis pathways and physiological concentrations of endogenous 3-T_1AM and T_0AM. However, also the published LC-MS/MS based serum concentrations detecting endogenous 3-T_1AM still differ between the groups working on TAM (Table 3).

4.2.1 Thyroidal biosynthesis of 3-T_1AM

In rodents, T_4 accounts for approximately 50% of the TH secreted by the thyroid gland. If 3-T_1AM is a major product of T_4 metabolism, a high 3-T_1AM concentration might be found in the thyroid gland. Using the LLE-based LC-MS/MS method for the isolation of TH and TAM from tissues, T_4, T_3, rT_3, 3,5-T_2, and 3-T_1AM were determined in perfused mouse thyroids suggesting thyroidal existence of 3-T_1AM. These data are concordant with other publications

showing thyroidal 3-T_1AM, but in non-perfused tissue samples (Geraci, 2008; Saba, et al., 2010). 3-T_1AM is taken up by the differentiated thyroid follicular cell line derived from normal rat thyroid FRTL-5 cells as shown in this work and independently by Agretti, et al. (Agretti, et al., 2011). Administration of 1 µM 3-T_1AM for four hours results in the catabolism of 3-T_1AM to T_0AM, T_1Ac and T_0Ac confirming the presence of DIO and MAO activity in these thyroid cells. FRTL-5 cells are able to accumulate exogenous T_4 and to convert it into T_3 (other catabolites were not detected) proving that T_4 is really taken up into the FRTL-5 cell. However, the complete deiodination and decarboxylation of T_4 into 3-T_1AM was not observed in FRTL-5 cells after four hours incubation of 1 µM T_4 (Agretti, et al., 2011) suggesting that these cells might not express the desired TH decarboxylase.

4.2.2 Extrathyroidal production of 3-T_1AM

Still, no evidence for TAM *de novo* biosynthesis in the thyroid gland from tyrosine or tyramine and iodination of the iodine-free T_0AM has been presented as an alternative hypothesis of the biosynthesis pathway. However, the complex biosynthesis of diphenlyether-coupled (iodo-)thyronine molecules has so far only be shown *in vivo* in the thyroid gland, which still remains the major source of TH in humans and higher animals. Usually, the thyroid gland has been considered as the only source of TH production. In humans it is assumed, that approximately 80% of circulating T_4 originates directly from the thyroid (Laurberg, 1984). Several TH metabolites (T_4, T_3, rT_3, 3,5-T_2, and 3-T_1AM) were detected in perfused mouse tissues using LC-MS/MS showing extrathyroidal existence of TH metabolites and 3-T_1AM. Nevertheless, a thyroidal production cannot be totally excluded since 3-T_1AM is present in murine thyroids. Therefore, 3-T_1AM serum levels were investigated in a thyroid cancer cohort. In these TSH suppressed T_4-substituted patients all of whom lack functional thyroid tissue after surgery and/or radioiodine therapy, 3-T_1AM serum levels are detectable at equal or even higher levels compared to healthy controls. This underlines the theory of extrathyroidal 3-T_1AM production from exogenous T_4 supplements. This assumption is supported by the fact that under TSH suppressive therapy, where higher than normal T_4 serum values are reached, also 3-T_1AM levels are higher than in controls. In T_4 substituted thyroid cancer patients, neither age-dependent variations of serum 3-T_1AM serum were observed nor did females and males show different 3-T_1AM levels, which is compatible with the fact that mean age, body weight, and body mass index were not significantly different between women and

men. In addition, in T_4-substituted patients with pituitary insufficiency leading to an inactive thyroid gland, 3-T_1AM is still detected without a decrease six days after drug withdrawal, while fT_4 and fT_3 levels are already clearly diminished as expected. The 3-T_1AM serum profile during T_4 withdrawal suggests either a rather long half-life or persisting 3-T_1AM release into serum from intracellular TH precursors or stores. Further kinetic studies after administration of classical TH versus 3-T_1AM need to be done and might also give indication on the source of 3-T_1AM biosynthesis.

Every now and then, the concept of extrathyroidal TH synthesis has been discussed and tested in several experiments of several authors providing solid evidence for the process of a low-level TH generation in tissues other than the thyroid gland (Cuervo Munoz, 1958; Evans, et al., 1966; Obregon, et al., 1981; Purves and Griesbach, 1946; Taurog and Evans, 1967). Nowadays, sensitive LC-MS/MS methods are used to investigate TH kinetics and metabolism trying to answer the question of extrathyroidal TH formation (Nagao, et al., 2011). However, most thyroidologists are still skeptical since some experiments are only *in vitro* and TH synthesis is too unique to take place in every cell or tissue. This hypothesis of extrathyroidal TH synthesis is based on the observations that thyroidectomized animals still have low levels of TH. The reason for this might be due to TH production by extrathyroidal tissues, enhancement of enterohepatic recirculation and repression of TH degradation or incomplete removal of the thyroid gland. Taurog, et al. demonstrated in thyroidectomized rats that the biological actions concerning growth, metabolic rate, heart rate, and pituitary, adrenal, and reproductive function of iodide are attributable to the extrathyroidal formation of small quantities of T_4 (Taurog and Evans, 1967). Later, they presented the existence of T_4 in the plasma of the thyroidectomized rats that received 5 mg of iodide daily by scans of paper chromatograms (Taurog and Evans, 1967). Obregon, et al. showed low levels of T_4 and T_3 by specific RIAs in tissues (liver, kidney, brain, heart, and hindleg muscle) from thyroidectomized rats that had received a single dose of ^{131}I long after these hormones had disappeared from the blood circulation (Obregon, et al., 1981). However, these observations were never followed up to clarify the source of extrathyroidal TH.

In recent years, Meischl, et al. reported that rat H9c2 cardiomyocytes express all components essential for TH biosynthesis. Therefore, they used spotted oligonucleotide microarrays to analyze the cardiac mRNA expression in normal, hypertrophic, and failing ventricular tissue. They found low but significant expression of Tg, NIS, pendrin, TPO, and TSHr in

H9c2 cells which also expressed DUOX1 and DUOX2 at the mRNA level and at the protein level (Meischl, et al., 2008). Moreover, Meischl, et al. showed intracrine TH production under their experimental conditions (Meischl, et al., 2008). They incubated H9c2 cells for 88 h in the presence of 50 µCi ^{125}I/ml and subsequently induced ischemia. SDS-PAGE analysis revealed that ^{125}I was incorporated exclusively into Tg. Moreover, a low but significant presence of free T_4 and free T_3 were found in these cardiomyocyte lysates using a modified RIA (Meischl, et al., 2008). However, high iodide doses were necessary to generate TH in the heart which does not possess the follicular structure that is important for the TH production of the thyroid gland. Taken together, rat H9c2 cardiomyocytes express the main proteins necessary for TH synthesis and are able to generate low levels of TH (Meischl, et al., 2008). Furthermore, 3-T_1AM production has been shown from T_3 in these cardiomyocytes providing evidence of TH as TAM precursor. In these experiments, H9c2 cells were incubated for 120 min in the presence of 1 µM 3-T_1AM. The concentration of 3-T_1AM in the incubation medium decreased exponentially while the 3-T_1AM concentration in cellular lysate increased. The catabolism of 3-T_1AM was also investigated in cell lysates and conditioned medium (Saba, et al., 2010). Only 2% of TA_1 was observed in the cell lysate, whereas neither T_0AM nor TA_0 was detected although these cells express DIO enzymes.

These findings motivated our Institute to work with this cell line. However, neither the endogenous expression of genes like TPO, NIS, and TSHR could be confirmed using PCR, nor the 3-T_1AM generation from T_3 as precursor. Nevertheless, Tg, as the macromolecular precursor of TH, was observed in these cells (unpublished data). Normally, Tg is thought to be uniquely expressed by thyroid epithelial cells. In the meanwhile, more publications appeared describing Tg expression in various cell types and tissues except the thyroid (Fernando, et al., 2012; Kaufmann, et al., 2004; Meischl, et al., 2008; Sellitti, et al., 2000).

4.2.3 The human AADC is not involved in thyronamine biosynthesis

Piehl, et al. have recently identified TAM as isoenzyme specific substrates of the DIO1, 2 and 3 and proposed several pathways for TAM biosynthesis (Piehl, et al., 2008). Interestingly, the DIO isoenzyme selectivity of TAM deiodination differs from that of TH. T_4AM is not a substrate for DIO1 and DIO2, and cannot be deiodinated to T_3AM, whereas T_4AM is a substrate for DIO3 and is readily deiodinated to rT_3AM. DIO1 and DIO2 can then sequentially deiodi-

nate rT_3AM to provide 3-T_1AM. This isozyme-selective processing suggests a specific biosynthetic pathway for endogenous 3-T_1AM production, with either T_4AM or rT_3AM serving as entry points into the pathway. These entry points would originate from decarboxylation of either T_4 or rT_3 (Piehl, et al., 2008; Scanlan, 2009), which has also been proposed as a possible precursor of 3-T_1AM by the group of Dratman (Dratman, 1974; Gompf, et al., 2010).

AADC promotes the decarboxylation of a variety of endogenous and synthetic aromatic amino acids (Zhu and Juorio, 1995). The functional significance of the broad substrate specificity of AADC is unknown. It has been postulated that AADC may have other functions such as in non-monoamine neurons, where it is also expressed (Zhu and Juorio, 1995). According to Dratman's initial hypothesis presented in 1974 (Dratman, 1974), AADC has been proposed several times as a candidate enzyme to convert T_4, rT_3 or other TH into TAM, such as 3-T_1AM and T_0AM (Braulke, et al., 2008; Doyle, et al., 2007; Dratman, 1974; Klieverik, et al., 2009; Pietsch, et al., 2007; Scanlan, 2009; Scanlan, et al., 2004; Wu, et al., 2005). Interestingly, TAM were re-discovered based upon the hypothesis that analogous to the formation of dopamine from L-DOPA, T_4 or a lower iodinated TH could be decarboxylated to various TAM (Cody, et al., 1984; Meyer and Hesch, 1983). Therefore, the possible role of human AADC in TAM biosynthesis from TH precursors was investigated as the second aim of this thesis. The data presented in this thesis reveal that the human recombinant AADC apparently does not catalyse the decarboxylation of TH under any of our experimental conditions challenging the initial hypothesis, while the AADC preparation actively generated dopamine from L-DOPA under the same conditions. In our experiments, the most important factors (temperature, time, pH) for enzymatic activity were varied. As reviewed by Bowsher and Henry (Bowsher and Henry, 1986), the rate of *in vitro* decarboxylation of aromatic amino acids is highly dependent on the pH of the reaction medium and on the type of substrate. However, varying pH, incubation temperature and incubation time showed no rT_3AM formation from rT_3, which is the favoured substrate in the Dratman hypothesis (Gompf, et al., 2010). Thus, it can be concluded that TH are not decarboxylated by human functional AADC. These *in vitro* observations are further supported by the finding that 3-T_1AM is detectable in plasma samples from patients with genetic *AADC* deficiency at similar concentrations as in healthy controls.

If the decarboxylation of TH takes only place coupled to deiodination, studies using a cell/ or tissue homogenate rich in DIO and AADC activity would be necessary. Highest levels of tis-

sue AADC are found in kidney, liver, and gastrointestinal tract (Bowsher and Henry, 1986), so that murine liver AADC could be used as an *ex vivo* enzyme source.

Figure 47: Biosynthesis of 3-T_1AM requires deiodination and decarboxylation.

3-T_1AM is structurally related to T_3 since it can be produced by deiodination and decarboxylation. However, human recombinant AADC is not the decarboxylating enzyme.

As the biosynthesis of monoamines mainly occurs in neural tissues, it is very surprising that AADC is highly abundant in kidney and liver where no monoamine biosynthesis is observed until now (Moreno, et al., 2008). Approximately 80 – 85% of T_3 is generated by DIO1, primarily in the liver and kidneys. Piehl, et al., tested the deiodination of every possible TH using murine liver homogenates as a DIO1 source as a positive control of the LC-MS/MS based DIO assay (Piehl, et al., 2008). The formation of 3-T_1AM or other TAM from murine liver homogenates incubated with all nine possible TH was not observed. However, TAM are substrates of metabolizing enzymes like SULT (Pietsch, et al., 2007), DIO (Piehl, et al., 2008), and MAO (Wood, et al., 2009). Although the physiological role of AADC in liver is not clear, these results indicate that liver AADC is not responsible for the production of TAM from TH. Although the AADC cannot completely be excluded as the decarboxylating enzyme *in vivo*, the findings do not support the hypothesis that AADC is a potent TH decarboxylase.

4.2.4 Searching for a new thyroid hormone decarboxylase

Since AADC may not be the TH decarboxylating enzyme, other candidate decarboxylases should be tested for their potential function to decarboxylate TH. Many of the identified decarboxylases have not been characterized so far and are orphan enzymes. If the new candidate enzyme is relatively similar to AADC, amino acid exchanges from big to small in the active site would be desirable. Such exchanges would create a void in the active site of the enzyme in comparison with AADC. This bigger active site is required, because 5-hydroxy-L-tryptophan, one of the biggest known AADC substrates, could be an exclusion size for AADC. TAM, exhibiting two aromatic rings, are even bigger than 5-hydroxy-L-tryptophan and,

consequently, need more space in the active site. A bioinformatic search for other candidate decarboxylases was performed in our Institute. An interesting new candidate was found which shows a very promising active site (Figure 48) while being highly similar to AADC.

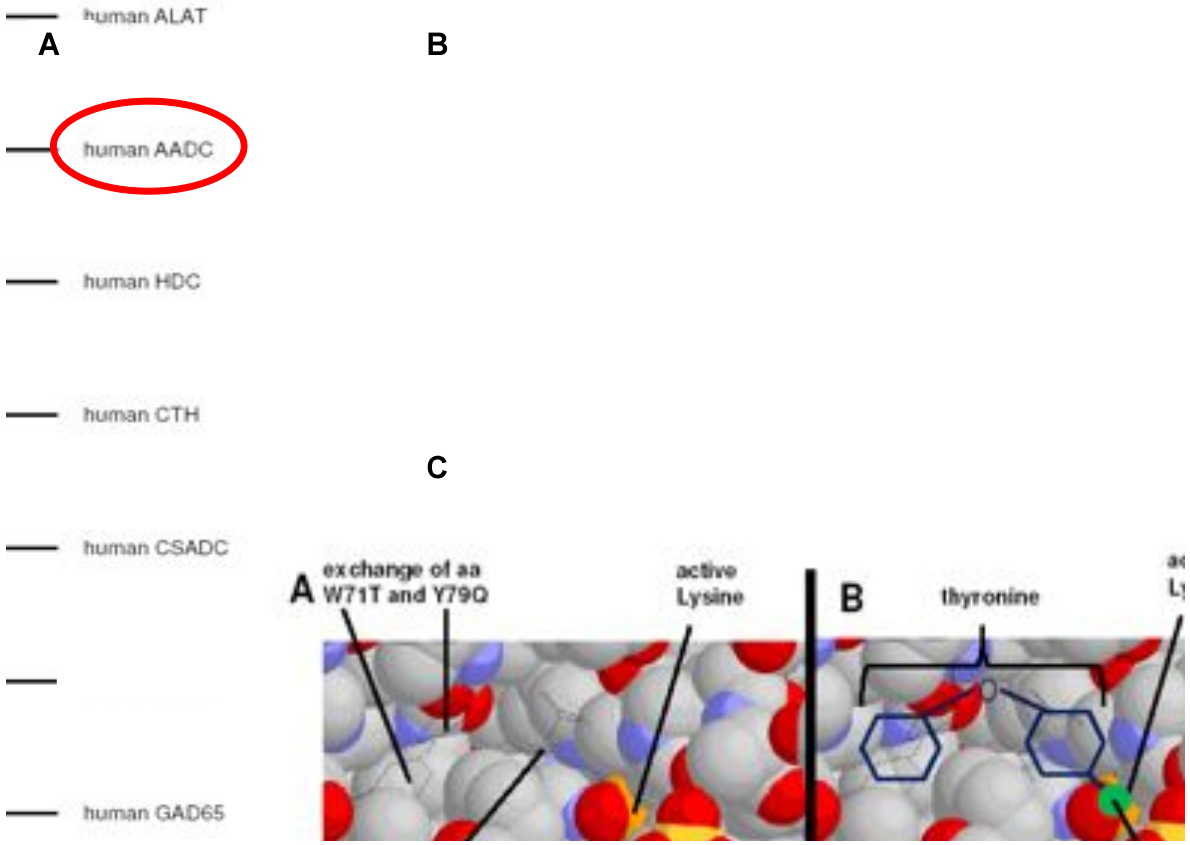


Figure 48: Bioinformatic search for a new candidate enzyme.

Phylogenetic tree based on all decarboxylating enzymes of human origin (A). Amino acid exchange in the active site between human AADC and the new candidate enzyme (B). Structure of AADC. Left the active site of AADC with bound inhibitor carbidopa and two exchanged amino acids in comparison to the new enzyme and right schematic visualization of possible binding of TH (C). Visualization using RasMol by Dr. Schweizer (Institut für Experimentelle Endokrinologie, Charité-Berlin, Germany).

This active site exhibits, in comparison to AADC, two amino acid exchanges, which create an additional free space. The substrate is orientated with its carboxyl group towards the catalytic active lysine residue, which would make decarboxylation feasible. Hence, this new candidate enzyme could be able to accommodate and decarboxylate TH. Further on, an expression profiling showed that this enzyme is mainly expressed in hypothalamus and brain, organs where pharmacological TAM effects play a role. Up to now, a low decarboxylation and deiodination rate of T_3 to 3-T_1AM was shown for the first time in H9C2 cardiomyocytes, these cells likely contain the elusive decarboxylase (Saba, et al., 2010). However, in rat FRTL-5

cells no TAM formation has been observed (Agretti, et al., 2011). Experiments using this and other cell models may provide further insight into the biosynthesis of TAM, which exhibit remarkable effects mainly counteracting those of the active T_3.

5 Conclusion and future perspective

After their initial discovery in the early 1950's, TAM are now back to the focus of basic and clinical research. Meanwhile, two representatives of TAM, namely 3-T_1AM and T_0AM, have been detected *in vivo* in several species (Hoefig, et al., 2011; Saba, et al., 2010; Scanlan, et al., 2004*)*. However, it is unknown if further representatives of TAM are present *in vivo*. Albeit their physiologic function remains elusive, administration of pharmacological doses of 3-T_1AM in rodent animal models elicited prompt effects, such as metabolic depression, hypothermia, negative chronotropy, negative inotropy, hyperglycemia, decreased respiratory quotient, ketonuria and reduction of fat mass as well as promising therapeutic potential in the experimental prophylaxis and treatment of stroke have already been demonstrated (reviewed in Piehl & Hoefig, et al. (Piehl, et al., 2011)). In terms of structure, TAM differ from TH only concerning the absence of the carboxylate group of the alanine side chain. However the physiological TAM concentration and the pathways and regulation of TAM biosynthesis are still under discussion. Both classical immunoassay based methods and novel LC-MS/MS analytics are suitable for TH quantification in body liquid and tissue samples. Nowadays, LC-MS/MS is used in research laboratories as gold standard method in endocrine analytics. However, clinical routine TH evaluation is still performed by immunoassays.

In conclusion, the present study supports the hypothesis that LC-MS/MS measurements are excellent to investigate several analytes in one sample in one analytical run. A novel SPE method followed by LC-MS/MS was established for the simultaneous detection and quantification of TH profiles in one serum sample independent from the species. The LLE extraction before LC-MS/MS analysis was successful to study tissue TH and TAM levels as well as the uptake of TH and TAM *in vitro*. Nevertheless, the determination of 3-T_1AM in serum using LC-MS/MS was not achieved in this work. Therefore, a highly specific CLIA was developed. This assay was used to study the biosynthesis of 3-T_1AM. Since 3-T_1AM levels are detectable in T_4-substituted patients in similar levels compared to healthy controls it was concluded that 3-T_1AM is mainly produced extrathyroidally albeit with a serum profile distinct from that of T_4 and T_3 during T_4 withdrawal. It has frequently been suggested that 3-T_1AM might be biosynthesized of TH by deiodination and decarboxylation of the alanine side chain. Piehl, et al. showed that TAM are substrates for all three DIO and suggested several possible pathways to convert T_4 into 3-T_1AM, but the decarboxylating enzyme which we have to postulate

remained elusive (Piehl, et al., 2008). The data presented in this work suggest that AADC is not involved in TAM biosynthesis. The goal to find the TH decarboxylase was not achieved but AADC, as the first candidate enzyme for TH decarboxylation, could be excluded. Therefore, TAM are probably formed by another decarboxylase since the conversion of T_3 into 3-T_1AM was shown in H9c2 cells (Saba, et al., 2010). Hence, H9c2 cells contain the needed DIO enzymes as well as the postulated decarboxylase to generate 3-T_1AM *in vitro*. Cell culture experiments with H9c2 cells and decarboxylase inhibitors would be one option to elucidate the TH decarboxylase. The LC-MS/MS therefore provides the best method to achieve this goal. The introduction of advanced and highly sensitive LC-MS/MS technology into laboratory practice will help to explain the TH metabolism.

The novel 3-T_1AM immunoassay constitutes an important new tool for addressing unresolved questions about the physiology of 3-T_1AM as a potential biomarker and its role in highly prevalent human diseases. In the future, comparative preanalytical experiments like the stated discoveries of Ghrelin are necessary for optimizing analytical conditions and setting up a standardized 3-T_1AM blood collection, blood storage, and measurement protocol. The comparison of different plasma matrices and factors like body weight or circadian rhythm are lacking for 3-T_1AM and in publication it is often unclear which kind of blood plasma source was used. To analyze free hormones less invasive, saliva, blood spots on filter paper, and urine are often used as a matrix of choice. Steroids (Al-Dujaili, et al., 2012) , TH (Higashi, et al., 2011) and trace elements like iodine (Grimm, et al., 2011) are measured from these matrices. Up to now, urinary or salivary 3-T_1AM concentrations have not been published. The measurement of the same serum samples using our immunoassay and LC-MS/MS are necessary to define the “true” total and free 3-T_1AM concentration. As mentioned above, it is important to keep in mind that immunological and LC-MS/MS methods might not correlate under some clinical conditions. Hence, more clinical studies and experiments are necessary to understand the physiological role and biosynthesis of TAM.

To better understand TAM metabolism, the introduction of the measurement of TH and TAM conjugates like sulfates and glucuronides using the LC-MS/MS would be helpful. One mechanism for termination of TH and TAM action other than glucuronidation or monoamine oxidation is through sulfation by cytosolic SULT (Pietsch, et al., 2007). Other TAM modifications are conceivable but not published at the moment (see Figure 4).

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List of acronyms and abbreviations

3-T_1AM	3-Iodothyronamine
5-HTP	5-Hydroxythyrptophan
AADC	Aromatic-L-amino acid decarboxylase
AC	Adenylylcyclase
apoB100	Apolipoprotein B100
BSA	Bovine serum albumin
cAMP	Cyclic Adenosinmonophospahte
CLIA	Chemiluminescence immunoassay
DAG	Diacylglycerol
Dehal1	Dehalogenase 1
Diac	Diiodothyroacetic acid
DIO	Deiodinase
DIT	Diiodotyrosine
DMEM	Dulbecco's Modiefied Eagle's Medium
DMSO	Dimethylsulfoxid
DUOX1/2	Dual oxidase 1/2
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELC	Ether link cleavage
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
fM	Femtomolar
GPCR	G-protein coupled receptor
H_2O_2	Hydrogen peroxide
HAT- DMEM	DMEM that contains hypoxanthine, aminopterin and thymidine
HDL	High-density lipoprotein
HPT	Hypothalamus- pituitary- thyroid axis
HRP	Horse radish peroxidase
HS	Horse serum
HT- DMEM	DMEM that contains hypoxanthine and thymidine
IP_3	Inositol 1,4,5-trisphosphate
kDA	Kilodalton
KLH	Keyhole limpet haemocyanin
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LLE	Liquid liquid extraction
OATP	Organic anion transporter
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
PLC	Phospholipase C
PLP	Pyridoxal-5-phosphate
MAb	Monoclonal antibody
MAO	Monoamine oxidase
MCT	Monocarboxylate transporter

MES	2-(*N*-morpholino)ethanesulfonic acid
MIT	Monoiodtyrosine
NIS	Sodium/iodide symporter
nM	Nanomolar
pM	Picomolar
RIA	Radioimmunoassay
RT	Room temperature
SLC	Solute carrier
SPE	Solid phase extraction
SRM	Selective reaction monitoring
SSAO	Semicarbazide-sensitive amine oxidase
SULT	Sulfotransferase
rT_3	Reverse T_3
T_3	Triiiodothyronine
T_4	Thyroxine
TAAR 1	Trace amine associated receptor 1
TAc	Iodothyroacetic acid
TAM	Thyronamine
TBG	Thyroxin binding globulin
Tetrac	Tetraiodothyroacetic acid
Tg	Thyroglobulin
TH	Thyroid hormones
TPO	Thyroid peroxidase
TR	Thyroid hormone receptor
TRE	Thyroid hormone responsive element
Triac	Triiodothyroacetic acid
TRH	TSH releasing hormone
TRHR	TSH releasing hormone receptor
TSH	Thyrotropin, thyroid-stimulating hormone
TSHR	Thyrotropin receptor
TTR	Transthyrretin
VLDL	Very low-density lipoprotein

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel „Establishment, validation and application of immunological and LC-MS/MS-based detection methods to study the role of human aromatic L-amino acid decarboxylase as an enzyme potentially involved in thyronamine biosynthesis“ selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel verfasst habe.

Des Weiteren erkläre ich meine Kenntnisnahme der dem angestrebten Verfahren zugrunde liegenden Promotionsordnung. Ich versichere, dass ich diese Arbeit weder in dieser noch in einer anderen Form bei einer anderen Prüfungsbehörde eingereicht habe und dass ich nicht im Besitz eines entsprechenden Doktorgrades bin.

Berlin, den ……..

……

Carolin Höfig