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HPLC-MS-MS analysis of thyroid hormones and iodotyrosines in knocked-out murine and natural human DEHAL-1 deficiency

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Abstract

Thyroid hormones (TH), namely 3,5,3'-triiodothyronine (T3) and its precursor thyroxine (T4), are key regulators of growth processes and development, and crucially control the energy metabolism. A proper availability of iodine within the thyroid is crucial for the synthesis of TH in order to maintain the homeostasis of circulating levels. The daily dietary iodine intake is not sufficient to sustain their synthesis and, in fact, most of the intra-thyroidal iodine is recycled through the activity of the Iodotyrosine Dehalogenase-1 (DEHAL-1) enzyme. Mono-iodotyrosine (MIT) and di-iodotyrosine (DIT) precursors produced in excess during TH synthesis, are deiodinated by DEHAL-1, leading to the production of I^- and Tyr that are recycled and reused within the thyroid. In the last decade, human mutations of *DEHAL1* causing the impairment of its catalytic activity were reported. The clinical picture caused by these mutations recapitulates the phenotype of what is known as Iodotyrosine deiodinase deficiency (ITDD), characterized by hypothyroidism, goitre and, if not treated during childhood, by intellectual impairments. A hallmark of ITDD resides in the increase of urinary and plasmatic MIT and DIT levels.

High-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS-MS) is a powerful technique characterized by high specificity and sensitivity. Even if clinical testing of TH is still performed using immunoassays, various HPLC-MS-MS methods to assay TH at low concentrations have been proposed. HPLC-MS-MS is now emerging as a powerful technique that complements traditional testing overcoming some of their limitations. Hitherto, only one HPLC-MS-MS method have been proposed in order to assay MIT and DIT levels in urine that has been used in the discovery of one of the most recent discovered human *DEHAL1* mutations.

The aim of my project was to develop a sensitive and robust HPLC-MS-MS method able to quantify MIT and DIT in urine and plasma, together with T3 and T4 in the latter case. The method was extensively validated and used to assay these molecules in urine and plasma collected from the novel *Dehal1* knock-out mouse. Our method played a crucial role in the biochemical characterization of this first mammalian model of defective iodine recycling through DEHAL-1 deletion. We detected increased urinary and plasmatic levels of MIT and DIT in the knock-out mice and we demonstrated the presence of elevated concentrations of both molecules even at the first stage of life. The influence of iodine availability was tested, showing that mice were still euthyroid when levels of dietary iodine were sufficiently high. In the presence of scarce iodine availability, coupled to the impaired ability to recycle iodine through DEHAL-1, knock-out mice became hypothyroid.

Our results demonstrated the importance of iodine availability in triggering hypothyroidism in the presence of ITDD.

The importance of a powerful technique able to detect MIT and DIT was demonstrated assaying these molecules in human urine collected from a consanguineous family with a diagnosed DEHAL-1 deficiency. Our HPLC-MS-MS method was able to detect elevated urinary levels of MIT and DIT in the index patient that was clinically diagnosed with ITDD showing goitre and hypothyroidism. Remarkably, we detected a massive increase of both molecules in urine collected from one of the brothers that was healthy at the time of sample collection but that developed hypothyroidism and goitre several years later.

In conclusion, our findings demonstrated the ability of our validated HPLC-MS-MS method to detect MIT, DIT together with T3 and T4 in urine and plasma sample. We showed the potential of MIT and DIT assay, especially in urine, for the early detection of hypothyroidism in DEHAL-1 deficiency. Considering the presence from the beginning of life, the detection of iodotyrosines could be potentially included in the human new-born screening for hypothyroidism.

Abbreviations

3-T1 = 3-iodo-L-Thyronine
AcK = Potassium acetate
ACN = Acetonitrile
AcOH = Acetic acid
AF-1 = Activation function 1
APCI = Atmospheric-pressure chemical ionization
API = Atmospheric-pressure ionization
APPI = Atmospheric-pressure photo ionization
BBB = Blood brain barrier
CAD = Collision gas
cAMP = Cyclic adenosine monophosphate
CE = Collision energy
CH_2Cl_2 = Methylene chloride
CI = Chemical ionization
CID = Collision-induced dissociation
$ClO4^-$ = Perchlorate
CP = Choroid plexus
CSF = Cerebrospinal fluid
CUR = Curtain gas
CxP = Collision exit potential
DBD = DNA binding domain
DEHAL = Iodotyrosine dehalogenase
DESI = Desorption electrospray ionization
DIO = Iodothyronine deiodinase
DIT = Di-iodotyrosine
DNT = 3,5-dinitro-L-Tyrosine
DP = Declustering potential
DUOX = Dual oxidase
ED = Equilibrium dialysis
EI = Electron ionization
EP = Entrance potential
ERK = Extracellular related kinase
ESI = Electrospray ionization

FA = Formic acid
FAB = Fast atom bombardment
FMN = Flavin mononucleotide
fTH = Free TH
GC = Gas chromatography
GC-MS = Gas chromatography coupled to mass spectrometry
GH = Growth hormone
GLUT = Glucose transporter
GS = Gas source
H_2O_2= hydrogen peroxide
HCl = Hydrochloric acid
HIF-1 = Hypoxia-inducible factor 1
HO = Homozygous
HPLC = High.performance liquid chromatography
HPLC-MS-MS = HPLC coupled to tandem mass spectrometry
HPT = Hypothalamic-pituitary-thyroid axis
HT = Heterozygous
I = Iodine
I^- = Iodide
I^+ = Iodinium
IQ1 = Focusing lens 1
IS = Internal standard
ISV = Ion spray voltage
ITDD = Iodotyrosine deiodinase deficiency
Ka = Association constant
KCN = Voltage-gated K^+ channels
Km = Michaelis-Menten constant
KO = Knock-out
KOMP = KO mouse project repository
LAT = L-Type amino acid transporters
LBD = Ligand binding domain
LC =Liquid Chromatography
LID = Low iodine diet
LOD = Limit of detection
LOQ = Limit of quantification

L-T4 = Levothyroxine
MALDI = Matrix-assisted laser desorption ionization
MAPK = Mitogen-activated protein kinase
MCT = Mono-carboxylate transporter
MeOH = Methanol
MIT = Mono-iodo tyrosine
MRM = Multiple reaction monitoring
MS = Mass spectrometry
MS-MS = Tandem mass spectrometry
NADPH = Nicotinamide adenine dinucleotide phosphate
NH_4OH = Ammonium hydroxide
NID = Normal iodine diet
NIS = Sodium-Iodide symporter
NTCP = Na^+/taurocholate co-transporter
OATP = Organic anion transporter polypeptide
PIK-3 = Phosphatidylinositol 3-kinase
PTU = 6n-propyl-2-thiouracil
PVN = Hypothalamic paraventricular nucleus
rT3 = 3,3',5'-triiodothyronine or reverse T3
RXR = Retinoid X receptor
SAGE = Serial analysis of gene expression
SCN^- = Thiocyanate
SD = Standard deviation
SEM = Standard error mean
SLC = Solute carrier family
SPE = Solid phase extraction
SRM = Selective reaction monitoring
T_0AM = Iodothyronamine
T_1AM = 3-iodothyronamine
T2 = Di-iodothyronine
T_2AM = Di-iodothyronamine
T3 = 3,5,3'-tri-iodothyronine
T_3AM = Tri-iodothyronamine
T4 = 3,3',5,5'-tetra-iodothyronine or Thyroxine
TA_1 = 3-iodo-thyroacetic acid

TAAR1 = Trace amine associated receptor 1
TBG = Thyroxine-binding globulin
TEM = Source temperature
Tetrac = 3,5,3’,5’-tetraiodothyroacetic acid
TG = Thyroglobulin
TH = Thyroid hormones
TPO = Thyroid peroxidase
TR = Thyroid receptor
TRH = Thyrotropin-releasing factor
Triac = 3,5,3’-triiodothyroacetic acid
TSH = Thyroid-stimulating hormone
TTH = Total TH
TTR = Transthyretin
UF = Ultrafiltration
UHPLC = Ultra-high pressure liquid chromatography
VLID = Very low iodine diet
WT = Wild-type

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Part I

Introduction

1 Thyroid hormones

1.1 Synthesis

Thyroid hormones (TH) play a key role in regulating growth, development and energy metabolism. They have the crucial function to control tissues differentiation, brain development, cardiovascular and skeletal homeostasis. Moreover, they are critically involved in the control of carbohydrates and lipid metabolism through the regulation of insulin sensitivity in the liver and the suppression of gluconeogenesis. TH regulate basal metabolic rate and adaptive thermogenesis, with a considerable effect on body weight [1]. With the term TH we refer to the iodinated tyrosine-based molecules 3,3',5,5'-tetra-iodothyronine (T4 or Thyroxine) and 3,5,3'-tri-iodothyronine (T3) (Figure 1). In humans, the thyroid produces mainly T4, considered as prohormone, since it is converted in peripheral tissues into the biologically active T3 which has a higher affinity for the nuclear thyroid hormone receptor (TR). The thyroid produces 20% of the circulating T3, in a ratio of 1:14 respect to T4 [2, 3].

Figure 1: *Chemical structure of T4 and T3. The two tyrosinic rings containing 4 and 3 atoms of iodine, respectively, are coupled together through an ether bridge.*

Levels of circulating TH are controlled by central and peripheral highly sensitive negative-feedback loop mechanisms. The hypothalamus and the pituitary regulate the production of TH by the thyroid, creating a system fundamental in preserving TH homeostasis, known as Hypothalamic-Pituitary-Thyroid axis (HPT). Thyrotropin-Releasing Factor (TRH) is a tripeptide (pGlu-His-ProNH2) secreted by hypothalamic paraventricular nucleus (PVN) in the hypothalamus [4]. The interaction between the secreted TRH and the G-protein coupled receptor TRH-1 on the pituitary thyrotrope

cells leads to an increase of intracellular cAMP that stimulates the synthesis and release of the pituitary Thyroid-Stimulating Hormone (TSH). Furthermore, TRH has also a role in the control of TSH activity, being important in its glycosylation [5]. TSH, known also as Thyrotropin, is a heterodimeric 28 KDa glycoprotein consisting of α and β subunits, secreted by pituitary thyrotrope cells. An appropriate glycosylation of TSH subunits is important for the interaction with its G-protein coupled TSH receptor on the thyroid follicular cell, which triggers the synthesis of TH [6]. The increase of circulating T3 and T4 controls the HPT through a highly sensitive negative feed-back mechanism, decreasing the synthesis of TRH and TSH at hypothalamus and pituitary gland, respectively (Figure 2) [7].

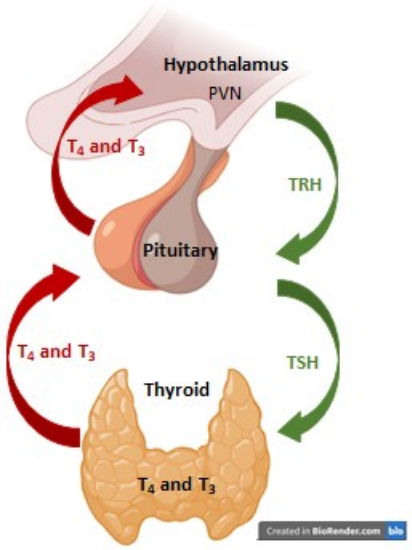

Figure 2: *Schematic view of the HPT axis and its negative feedback control mechanism. TSH synthesis is stimulated by hypothalamic TRH and inhibited by circulating TH, which are also responsible for the pituitary inhibition of TSH synthesis. Conversely, a decrease of circulating TH rapidly triggers the synthesis and release of both TRH and TSH, in order to normalize levels of T3 and T4.*

TH inhibits the synthesis of TRH and both subunits of TSH at a transcriptional level. Additionally, it has been reported the ability of TH to inhibit TSH post-transcriptional modifications and also its release [8]. Conversely, the reduction of circulating TH rapidly activates the secretion of TRH and, afterward, of TSH, leading to an increase TH synthesis and secretion in order to normalize circulating levels [9].

The other mechanism by which levels of TH are controlled takes place intracellularly and involves three members of the thioredoxin enzyme family, namely iodothyronine deiodinases (DIO) [3].

The iodotyrosine deiodinases type 1,2 and 3 (DIO1, DIO2 and DIO3, respectively) are responsible for the regulation of levels and the activity of thyroid hormones, through the removal of iodine from T4 precursors [10]. T4 enzymatic deiodination to form T3 mainly occurs in extrathyroidal tissues but, to a minor extent, also in the thyroid [11]. The three DIOs share an integral membrane nature and the common general structure comprehending a selenocysteine active site [12]. They comprise a single N-terminal, transmembrane segment connected to a larger globular cytosolic domain, containing the selenocysteine active site embedded in a thioredoxin-like fold [2]. The presence of this rare amino acid in the catalytic domain is critical for the reductive deiodination reaction: selenium provides the electrons needed for the elimination of iodine which is reduced to I- and increases substrate affinity and fast turnover rate. Finally, the oxidized enzyme is then reduced and regenerated through the protein thiols [13]. DIO1 and DIO2 catalyse the removal of the iodine at the outer ring (phenolic ring) of T4 to form T3, in a process resulting in its activation, whereas DIO3 is responsible for the removal of the iodine at the inner ring (tyrosyl ring) to form the inactive metabolite rT3 (3,3',5'-triiodothyronine or reverse T3) (Figure 3). The three isoforms possess different biochemical and regulatory features, exhibiting different tissue localization [11]. DIO1 is located in the plasma membrane and is able to convert T4 into T3 with a Km for T4 in the μM range. It is mainly expressed in liver, thyroid and kidneys and, as a matter of fact, it is not expressed in the central nervous system. DIO2 is the isoform responsible for the formation of T3 in the brain and it is also expressed in thyroid and in other key thyroid-responsive tissues, namely pituitary gland, skeletal muscles and brown adipose tissue [14]. DIO2 is located in the endoplasmic reticulum and its Km for T4 is in the nM range, meaning that T4 is a better substrate for DIO2 rather than DIO1 *in vitro*. DIO3, responsible for the inactivation of both T3 and T4, is present in the plasma membrane and it is mainly expressed in brain, placenta and pancreas [3]. DIO3 possesses a high affinity for T3 with a Km in the nM range and its activity is stimulated by developmental and disease signals [15].

Furthermore, the three isoforms respond to different inhibitors: while iopanoate is a common inhibitor of all three isoforms, 6n-propyl-2-thiouracil (PTU) is a selective DIO1 potent inhibitor and, from an historically point of view, played a fundamental role in the discovery of DIO2 [14, 16]. As already introduced above, most of the human circulating T3 is not produced by the thyroid gland, but it is the result of the deiodination activity in the extrathyroidal tissues, catalysed mainly by DIO2 and, with a minor extent, by DIO1 [17].

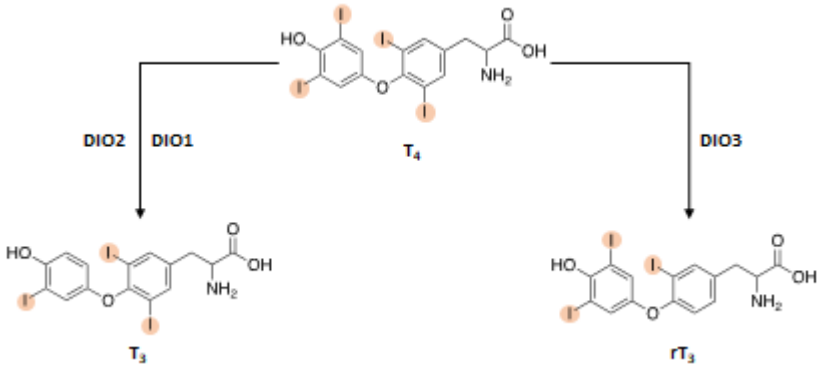

Figure 3: *TH deiodination reactions. T4 is activated by the catalytic activity of DIO1 and DIO2 to form T3 whereas the inactivation reaction leading to rT3 is catalysed by DIO3.*

T3 itself, is an important regulator of DIOs activity, stimulating the activity of DIO1 while having an opposite effect on DIO2 [18]. Hyperthyroidism increases DIO1 and decreases DIO2 expression, while the opposite is observed in hypothyroidism, in which the peripheral conversion T4 to T3 is enhanced by the DIO2 induction [3]. Moreover, T4 decreases the activity of DIO2 through post translational mechanisms, i.e. ubiquitin conjugation, contrary to DIO1 and DIO3 that are not susceptible to these reactions. Notably, *Dio1* and/or *Dio2* knock-out (KO) animal models have shown the ability of the thyroid to preserve circulating concentration of T3. The HPT is involved in this adaptive mechanism to maintain normal T3, since an increase of TSH serum level in response to reduced serum T4 has been observed [15].

The synthesis of TH is a complex process that comprises a series of tissue specific biochemical reactions. The functional unit of the thyroid for de-novo biosynthesis of TH is represented by thyroid follicles which are formed by a monolayer of follicular cells, called thyrocytes. The apical membrane of thyrocytes encloses the interior follicle lumen known as colloid, while the basolateral membrane faces the bloodstream [19]. The interior part of the follicles contains a large number of proteins among which, the most highly expressed, is an iodoglycoprotein called Thyroglobulin (TG). TG is a protein composed by two homodimers of ~330 KDa each and is the primary source of TH. In fact, it contains ~ 70 tyrosine residues with an averaging iodine content of 2.5-50 atoms of iodine per mol of TG and it can be itself a source of this essential element. TSH stimulates TG endocytic uptake into thyrocytes and its lysosomal degradation, resulting in the release of TH from the TG polypeptide backbone [20] (Figure 4).

Thyroidal iodine uptake from the bloodstream takes place on the basolateral membrane of thyro-

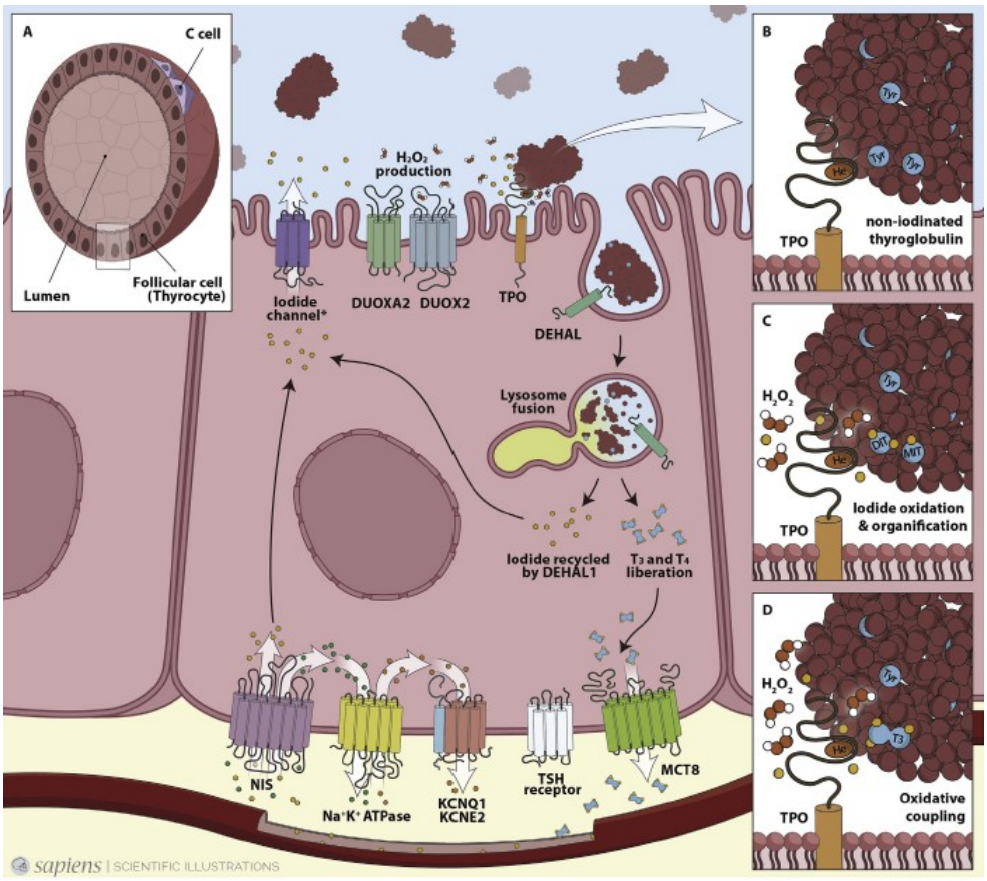

Figure 4: *Representation of the key proteins involved in the biosynthesis of thyroid hormones. A. Structure of thyroid follicles showing C cells involved in the production of calcitonin, important in the control of circulating Ca2+ levels; B,C and D. Main steps involved in TPO and TG activity leading to oxidative coupling and synthesis of TH (Adapted from Carvalho et al. [19]).*

cytes driven by the sodium-iodide symporter (NIS). NIS is a 13-segments transmembrane glycoprotein belonging to the solute carrier family 5 (SLC5A5) which transports 2 ions of Na^+ per each I^- ion [21]. The active transport of I^- against its electrochemical gradient inside the cytoplasm, relies on the activity of another important transmembrane protein, namely the Na^+/K^+-ATPase pump. With its activity, NIS is able to concentrate I^- around 30-60 folds in the cytoplasm of thyrocytes. This ensures that most of the ingested iodide, a scarce element in the environment, is accumulated in the thyroid and available for the synthesis of TH [22]. NIS transcription, translation and activation are stimulated by TSH through the activation of the G-protein coupled TSH receptor [23, 24]. Conversely, the transport of I^- inside the thyrocyte is blocked by competitor NIS inhibitors, i.e. perchlorate ($ClO4^-$) and thiocyanate (SCN^-) [19]. I^- itself is considered a main factor in controlling the accumulation of I^-. In 1948, Wolff and Chaikoff reported that high levels of iodide were able to

reduce its transport and accumulation in rat thyroid. This effect became known as “Wolff-Chaikoff effect”[25].

However, even in presence of high levels of I^-, TH biosynthesis can be restored after ~ 2 days through an adaptive mechanism mediated by the inhibition of I^- transport and known as “escape from Wolff-Chaikoff effect” [26, 27]. The electrochemical equilibrium is then restored via the presence on the basolateral membrane of two voltage-gated K^+ channels, namely KCNQ1 and KCNE2. These ion channels are responsible for the efflux of K^+ outside the cell and their key role in thyroid function has been reported [28].

Once inside the cytoplasm, I is transported to the thyrocyte apical membrane and finally towards the follicle lumen. This transport, known as “iodide efflux”, is mediated by a number of apical channels among which Pendrin was the first one discovered [29]. The rapid translocation of Pendrin toward the apical membrane is regulated by TSH through post-translational mechanisms, resulting in an increase of iodide efflux [30]. More recently, the important role in the process of iodide efflux of the chloride channel ClC5 and the calcium-activated chloride channel Anoctamin-1, has been demonstrated [31, 32].

I^- ions inside the lumen need to be oxidized in order to be integrated and bound to the tyrosyl residues of TG [19]. This oxidation reaction is catalyzed by the key enzyme of the TH biosynthesis, namely thyroid peroxidase (TPO). This is a glycosylated oxidoreductase with the fundamental role in iodination and coupling of iodotyrosines residues on TG, leading to T3 and T4 synthesis. The heme-containing active site and most parts of TPO are situated in the follicular lumen and form the extracellular process responsible for the enzymatic activity, whereas the small transmembrane C-terminal region anchors it at the apical membrane of thyrocytes [33, 34, 35]. The oxidation reaction of iodide molecules makes use of hydrogen peroxide (H_2O_2) generated by dual oxidase enzymes, namely DUOX1 and DUOX2. These Ca^{2+}-dependent glycoflavoproteins are members of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family and they are located on the apical surfaces of thyrocytes, together with their activators DUOXA1 and DUOXA2 [36, 37]. H_2O_2 is responsible for the oxidation of TPO, so that it can oxidize I^- to form iodinium (I^+) and hypo-iodite (IOH^-) ions. These very reactive iodine species bind to tyrosyl residues on the TG backbone to form mono-iodo tyrosine (MIT) and di-iodo tyrosine (DIT) [19].

The next step leads to the synthesis of T3 and T4, a process called thyroid hormonogenic coupling reaction. The exact mechanism of this process is still poorly understood, even if it has been hypoth-

esized the involvement of a free radical intermediate or an ionic interaction. However, hormonogenic coupling involves the donation of iodophenoxyl group from MIT or DIT called “donor”, to a DIT residue called “acceptor”, producing T3 and T4, respectively, and leaving a dehydroalanine in the “donor” position, as a side products of the reaction [38]. The analysis of kinetic models of iodination and hormonogenic coupling reactions revealed that only iodotyrosines properly oriented in the space can form T3 and T4. Moreover, they showed that iodinated tyrosyl residues preferentially admit the biosynthesis of T4 that, as already mentioned, is the main product of the thyroid [39]. Only 2 to 8 molecules of TH are formed from the 25-30 Tyr residues iodinated by TPO and, in normal conditions, TG roughly contains 2.5 residues of T4 and 0.7 of T3 [19]. Intrathyroidal T3 biosynthesis, formed by the coupling of a MIT and a DIT residue, follows the same pathway as T4. Noteworthy, most of the circulating T3 is not synthetized through T4 deiodination on TG, but it is the result of the DIO1 and DIO2 peripheral actions stimulated by TSH [10].

Synthetized TH bound onto TG are stored in the colloid until the increase of TSH levels promotes its internalization into thyrocytes via endocytosis processes. T3 and T4, together with uncoupled MIT and DIT, are released from the newly formed lysosomes by the proteolytic activity of endopeptidases. Free released MIT and DIT molecules, are enzymatically deiodinated to obtain I^- and Tyr residues that can be reused in the biosynthesis of TH. This important recycling process is catalyzed by an enzyme known as iodotyrosine dehalogenase (DEHAL-1) [40] which will be extensively described in the Paragraph 1.5.

1.2 Transport

Synthetized TH need to be released into the bloodstream, with a process that, for a long time, was believed to occur by a passive diffusion mechanism considering the lipophilic structure of TH. Lately, it has been described that TH reach the bloodstream through a series of transporters present on the basolateral plasma membrane of thyrocytes.

TH transporters belong to different families of solute carriers, they are not specific for TH and are widely expressed among tissues other than the thyroid [41]. Mono-carboxylate transporters (MCT) are a family of 14 transporters, among which MCT8 and MCT10 are sodium and proton independent TH transporters that mediate their transport through the plasma membrane [42, 43]. MCT8 is a highly specific TH transporter whereas MCT10 is an aromatic amino acids transporter possessing a slightly higher efficiency at transporting T3, but lower efficiency at transporting T4 [41, 44]. MCT8 has a particular importance for TH uptake into the brain, as revealed by the identi-

fication of an X-linked mutation leading to a syndrome characterized by psychomotor retardation, known as Allan-Herndon-Dudley syndrome [45, 46]. Even if circulating T3 levels are elevated, this neurodevelopment disorder is characterized by low intracerebral TH concentration, leading to a cerebral hypothyroid state during brain development [47]. MCT8 is expressed in many tissues in addition to thyroid (liver, heart, kidney, placenta, intestine, thyroid, and brain) and in distinct areas within the brain with an important role for T3 transport across the blood-brain barrier (BBB). Pathogenic mutations in MCT8 encoding gene have been detected in several diseases, indicating a pathophysiological role for TH transport [41]. In the last two decades, KO models have been designed to better understand the role of MCT8 in different tissues and the pathological implication of its mutations (the reader can refer to some excellent reviews [48, 49]). Liver Na^+/taurocholate cotransporter (NTCP) is a seven transmembrane domains glycoprotein involved in the enterohepatic circulation of bile acid. Belonging to the solute carrier gene family (SLC10A), these proteins recognize TH sulphate derivatives which are transported to the liver where DIO1 is responsible for the deiodination and rapid degradation of iodothyronine sulphate [50]. The L-Type amino acid transporters (LAT) are heterodimeric protein comprised of a 12 transmembrane light chain domain and single transmembrane glycosylated heavy chain domain, generally involved in the transport of neutral amino acids. LAT1 and LAT2 are the two isoforms involved in TH transport, with differential affinity: in fact, LAT1 transports T3 more efficiently than LAT2 [51, 52]. LAT1 and LAT2 have different tissue distribution, with the first one primarily distributed in brain, placenta and tumors, and the latter one primarily distributed in kidney, colon and intestine [53]. Organic anion transporter polypeptide (OATP) is a family of 12 transmembrane domain proteins involved in the transport of a series of amphipathic compounds, like steroids, anionic oligopeptides, bile salts and drugs. Up to seven members of this family are able to transport TH, among which OATP1C1 shows higher specificity to transport T3, rT3, T4 and sulphate T4 derivatives. It is expressed in several tissues but its physiological relevance is still unknown [41, 48]. Since TH are highly hydrophobic molecules, several carrier proteins are responsible for their transport and distribution through the bloodstream and cellular compartments [11]. Almost 95% of circulating TH are bound to thyroxine-binding globulin (TBG), transthyretin (TTR) and albumin, whereas the rest 5% is bound to minor carrier proteins, like lipoproteins or, with a minor extent, can be available in the circulation as free TH (fTH). Blood carrier proteins most probably have the function to ensure a constant availability of TH to the cells and tissues, avoiding urinary loss and controlling TH levels during abnormal production and degradation.

TBG is a glycoprotein synthetized in the liver made of a single 56 KDa polypeptide chain. It has only one binding site for TH, with an association constant Ka of $1x10^{10}$ M^{-1} and $4.6x10^{8}$ M^{-1} for

T4 and T3, respectively. Notably, the binding of T4 to TBG induces conformational changes that increase its stability. Since it carries the major part of TH (~ 70%), qualitative and quantitative abnormalities of TBG have a significant impact on total circulating TH levels [54, 55].

TTR, formerly known as thyroxine-binding pre-albumin, is a 55 KDa protein that circulates in blood as a stable homotetramer of identical subunits of 127 amino acids each [56]. Subsequently to the discovery of its TH binding properties, it has been demonstrated that TTR exists also in part as a complex with retinol binding protein, with a role in the transport of Vitamin A [57]. Only one of two TH binding sites is occupied and it has a Ka of $2x10^{8}$ M^{-1} and $1x10^{6}$ M^{-1} for T4 and T3, respectively. The lower affinity for TH respect to TBG, allows their rapid dissociation from TTR which, therefore, seems to be associated with the immediate delivery of T3 and T4 [55]. TTR carries 10 to 15 % of protein bound T4 and it is mostly synthetized in liver and, with a minor extent, in the central nervous system. It has an important role in the transport and delivery of T4 in the cerebrospinal fluid (CSF), where it is the main TH transporting protein [58].

Albumin binds ~5% of circulating TH with a lower affinity respect to TBG and TTR: in fact, its Ka $1.5x10^{6}$ M^{-1} and $2x10^{5}$ M^{-1} for T4 and T3, respectively [55]. Despite these low affinities, the relative contribution in TH transport is still significant because of the high amount of albumin that circulates in human serum. It has been proposed an albumin role as a fast TH resource during the rapid exchange in capillary transits [59].

TH play a crucial role in the brain development and maintenance of its adult functions. In fact, TH deficiency in fetal and neonatal periods results in cretinism characterized by mental retardation, deafness and ataxia, whereas in adult, can cause impairment of motor skills, intellectual defects and spasticity. For these reasons, adequate levels of TH in the brain are essential and can reach the brain by crossing the BBB, the highly selective permeability barrier which separates the bloodstream from the brain extracellular fluids. T4 is thought to be the primary TH crossing the BBB to be then locally converted into T3 by DIO2 in the hypothalamic astrocytes and tanycytes [60]. TH can cross the BBB via the already mentioned specific transporters such as MCT8 or OATP1C1 or can also indirectly reach the brain via the blood-CSF barrier. Choroid plexus (CP) is a highly vascularized structure part of the brain ventricular part, responsible for the synthesis of CSF and TTR, which is the main T4 transporter in this fluid. TTR plays a pivotal role in the delivery of T4 from plasma into CP and in its subsequent transport into CSF, offering an optimal exchange to deliver T4 into the brain [61, 62].

1.3 Mechanism of action

TH are essential for many diverse processes, like tissues development and the regulation of cellular metabolism. The effects of TH are mediated by two mechanisms: a genomic one, in which TH interacts with nuclear TR, and a nongenomic one, with a rapid onset, in which TH interacts with plasma membranes and other receptors [63].

TR are nuclear receptors that act as ligand-dependent transcription factors, able to modulate the transcription of target genes when activated by TH. In fact, the binding of T3 to TR can increase or decrease the transcription rate of target genes. In the nucleus, TR can constitutively bind to specific regions located in the promoter region of TH target genes, known as thyroid hormone response element (TRE) [64]. It has been shown that TR are still bound to TRE even in the absence of TH, repressing or silencing the basal transcription of the positive regulated target genes [65]. In the absence of its ligand, TR can be found as monomer, homodimers if coupled with another TR, or heterodimers, mainly coupled with retinoid X receptor (RXR) [66] (Figure 5).

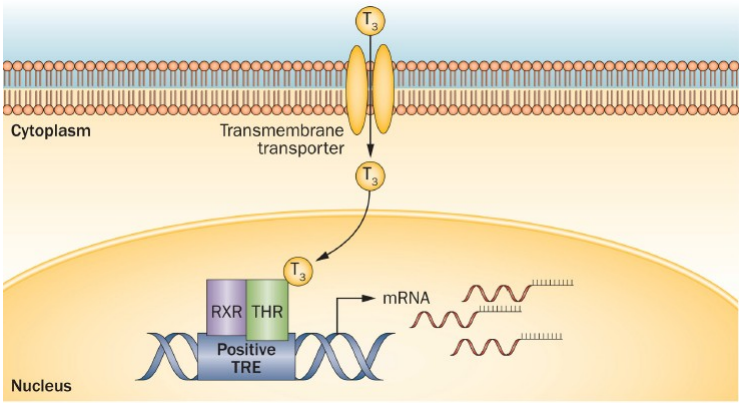

Figure 5: *TH genomic mechanism of action (Adapted from Ortiga-Carvalho et al. [64])*

The heterodimer TR-RXR appears to be the favoured conformation to bind the DNA, even if the *in vivo* functions and the roles of these complexes are still under investigation and remain to be determined [67].

The two genes TRA and TRB encode for the two nuclear receptors, TRα and TRβ, respectively. Not all the mammalian TR protein act as a nuclear receptor and, in fact, other proteins can be synthetized from alternative splicing, but their physiological roles are still unknown. The four isoforms acting as functional receptors are TRα1, TRβ1, TRβ2, and TRβ3. These functional isoforms

showed different patterns of expression in development and in adult tissues: TRα1 and TRβ1 are widely expressed, with the first one mostly expressed in brain, heart, skeletal muscle, and brown adipose tissue, and the latter one in brain, liver, and kidney. TRβ2 is predominantly expressed in pituitary gland, hypothalamus, and cochlea, whereas TRβ3 is mainly expressed in kidney, liver, and lung, and seemed to be functional in rat [68, 69]. TRβ is the responsible of most of TH effects on metabolism and, from a pharmacological point of view, also an ideal target to treat metabolic disorders, mainly lipid-related, or brain diseases. An ideal thyromimetic drug, should possess a high TRβ selectivity, in order to avoid any adverse effects on bone and heart [70].

TR are member of the highly conserved nuclear receptor superfamily and consist of a single peptide that is folded into three different functional domains: an amino terminal domain (A/B) which also contains the activation function 1 region (AF-1) involved in transactivation; a central DNA binding domain (DBD) containing two "zinc fingers", critically important in sequence-recognition on the TRE; and a carboxyl terminal ligand binding domain (LBD) which determines the specificity and high affinity of the receptor for TH ligands [63]. An additional linker region between DBD and LBD domains contributes to activation, repression and corepressors interaction and it is important for DNA and ligand binding [71].

Nongenomic effects of TH do not involve the classical concept of TR mediated TH action. They do not require gene transcription and protein synthesis but can include modulation of gene transcription. The mechanisms of several nongenomic actions depend upon signal transduction system and can involve novel TH membrane receptors, extranuclear TRβ or truncated isoforms of TR α [63, 72].

A novel receptor unrelated to classical TR exists on the integrin αVβ3, a plasma membrane structural protein highly expressed in tumour cells and dividing endothelial cells. Integrin αVβ3 has no structural homology with TR and TH binding takes place nearby the Arg-Gly-Asp recognition site where are located the two binding sites [73]. Binding of T3 activates phosphatidylinositol 3-kinase (PIK-3), specifically driving TRα into the nucleus and promoting transcription of hypoxia-inducible factor 1 (HIF-1) gene. TH can also interact with the integrin receptor and activates the extracellular related kinase 1 and 2 (ERK-1 and ERK-2) responsible for the transduction of TH signals in cancer cells. Moreover, interaction of both T3 and T4 with integrin αVβ3 is involved in their demineralizing action [63].

TH can rapidly stimulate PIK-3 and Rac activity on the plasma membrane through the interaction

with TRβ. The result is an activation of voltage-dependent potassium channels with a decrease of excitability and reduction of hormone secretion [74]. The activation of PIK-3 mediated by T3 has also direct and indirect effects on the transcriptional increase HIF-1, glucose transporter 1 (GLUT-1), and MCT4 transporter [65]. Both T3 and T4 can stimulate mitogen-activated protein kinase (MAPK) activity increasing angiogenesis. The activation of MAPK leads to phosphorylation of the tumour protein p53 with a final decrease of its transcriptional activity. In the nucleus, activated kinase forms a complex with TR β and phosphorylate its serine. T3 is also responsible of many nongenomic actions on plasma membrane proteins, contributing to basal activity of some ion pumps such as Ca^{2+}-ATPase, Na^{+}/K^{+}-ATPase and Na^{+}/H^{+} antiporter [75].

Recent studies on astrocyte cells, demonstrated the involvement of truncated TRα isoforms, namely TRΔα1 TRΔα2, in the regulation of actin cytoskeleton modelling which has a key role in the developmental program of the brain [72]. In response to T3, truncated TRα isoforms have shown to be imported into mitochondrial inner membrane where they are able to directly stimulate oxidative phosphorylation processes [65].

1.4 Peripheral metabolism

As already discussed above, the deiodination reaction of T4 to form the active T3 or the inactive rT3 is catalysed by DIO enzymes that are important in the control of TH levels. Once inside the cell, TH can undergo to tissue-specific metabolism, which can involve further deiodination, deamination, decarboxylation, sulfonation and conjugation reactions (Figure 6).

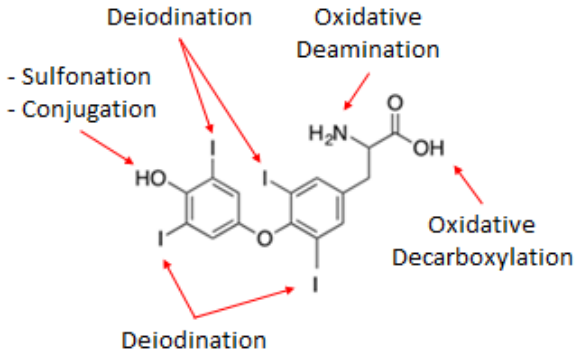

Figure 6: *Schematic overview of the classical pathways of TH.*

T3 and rT3 can be subjected to additional deiodination reactions, to form 3,5-diiodothyronine (3,5-T2), 3,3'-diiodothyronine (3,3'-T2), 3',5'-diiodothyronine (3',5'-T2) and 3-iodo-L-Thyronine (3-

T1). 3,3'-T2 and 3',5'-T2 demonstrated no remarkable activities, whereas 3,5-T2 proved the ability to bind to TR and have been studied for a long time by researchers. 3,5-T2 actions are mediated by the TR classical interaction but also through rapid effects at the cell membrane and mitochondria [76]. *In vivo* animal experimental models showed the ability of administered 3,5-T2 to alter the expression of canonical T3 regulated genes in various tissues, whereas, in pituitary models, it was able to suppress TSH and stimulate GH [77]. Animal models showed that the parental administration of 3,5-T2 was able to modulate energy metabolism, probably through the stimulation of liver fatty acid oxidation into mitochondria [78, 79]. Moreover, 3,5-T2 administration was able to prevent body weight gain, liver adiposity, hyperlipidaemia and insulin resistance, without any sign of thyrotoxicosis [76]. Acute 3,5-T2 exposition in an isolated rat heart model, showed the ability to increase oxidative metabolism without affecting cardiac contractility [80]. The physiological relevance of 3,5-T2 and 3,3'-T2 remains to be determined, however their presence in serum has been confirmed through the application of immunoassays and accurate high performance liquid chromatography tandem mass spectrometry (HPLC-MS-MS) methods. Nowadays, their actual serum concentrations are still debated and no clinical applications of their detection have been reported [81, 82, 83].

DIO are also able to deiodinate deaminated TH metabolites, namely 3,5,3',5'-tetraiodothyroacetic acid (Tetrac) and 3,5,3'-triiodothyroacetic acid (Triac). Tetrac is produced by deamination of T4 and its presence in human serum have been confirmed at low nM concentrations. Triac can be produced either by deiodination of Tetrac or by deamination of T3 and, despite its importance in pathophysiological cell-specific actions, distorted serum concentrations have been reported. They are both transported by TTR in serum and they act as thyromimetic compounds lowering TSH concentrations. Tetrac binds to TR onto integrin αVβ3 and elevated concentrations of this compound have been found in Grave's disease patients. Triac has a potent T3-mimetic activity showing a high affinity for TRβ and it has been described to be a substrate for MCT8 transporter [77].

Thyronamines are a novel class of endogenous iodothyronine-like signalling compounds that are structurally related to TH, differing only for the absence of the carboxylate group on the alanine side chain. Thyronamines differ for the number and position of iodine atoms, they are designated with T_xAM and their nomenclature is analogous to TH, where x is the number of iodine atoms [84]. Hitherto, only two members of this class have been detected in human serum using HPLC-MS-MS, namely Thyronamine (T_0AM) and 3-iodothyronamine (T_1AM) [85, 86, 87, 88]. In 2004, the discovery of a transient hypothermia effects after the administration of T_1AM in rats, spurred the researcher in studying this novel class of compounds in order to elucidate their biosyn-

thetic pathways, mechanisms of action and pathophysiological effects. In the pioneering paper by Scanlan *et al*., it was also demonstrated that the rapid onset of T_1AM effects was not compatible with the classical TH nuclear mechanisms, but more suitable with a G-coupled receptor. The suggested receptor responsible of T_1AM effects was identified in the trace amine associated receptor 1 (TAAR1). However, mechanisms of T_1AM actions can include other membrane receptors and intracellular targets which are still debated and not clear [86, 85, 77]. The mechanism of endogenous T_1AM biosynthesis is still uncertain. One of the putative mechanisms involves the deiodination of T3 or T4 to form T2, which is decarboxylated to form di-iodo-thyronamine (T_2AM) and, then, further deiodinated to form T_1AM [89]. Additionally to the already cited hypothermic effects, *in vivo* and *in vitro* models showed the ability of administered T_1AM to reduce cardiac inotropic and chronotropic effects [90, 91, 92], to protect during in ischemia reperfusion models [93, 94], to induce acute metabolic responses with actions on carbohydrates and lipids [95, 96, 97, 98] and it is emerging as a possible modulator of noradrenergic, dopaminergic and histaminergic systems [99]. T_1AM and thyronamines can undergo several metabolic reactions such as sulfonation, glucuronidation and acetylation whose effects are still under investigation [100].

In most tissues, the major metabolite of exogenous T_1AM and T_3AM are the corresponding acidic compounds, namely 3-iodo-thyroacetic acid (TA_1) and the already mentioned Triac, respectively. The mechanism for the biosynthesis of TA_1 is still under debate. A putative pathway was postulated by Lorenzini *et al*. and involved the oxidative deamination of T_1AM to form the corresponding acid TA_1 through an aldehydic intermediate [101]. The biological role of TA_1 is still unknown, even if an activity on the histaminergic system has been reported [102, 103].
Moreover, the phenolic hydroxyl group of TH can undergo conjugation reactions with the formation of sulfonated and glucuronated metabolites. Sulfation reactions of T4, T3, T2 and thyronamines have been reported as well, catalysed by sulfotransferase enzymes. Interestingly, sulfonated TH have a high affinity for DIO1 and this modification is a critical step to enhance the deiodination and inactivation of T4, T3 and T2 [17]. Interestingly, sulphate T3 can represent a reservoir of inactive hormone and might undergo enterohepatic recycling, whereas sulphate T2 might be useful as a biomarker for fetal thyroid function considering its secretion into maternal circulation through the placenta. Sulphated thyronamines have been investigated as well, but their biological roles and relevance are still unclear [77]. Sulfonation and glucuronidation are part of the so-called phase II detoxification reactions and they are involved in the inactivation and excretion of TH. The general aim of these reactions is to increase the solubility of TH in water and facilitate their excretion through bile and/or urine [84].

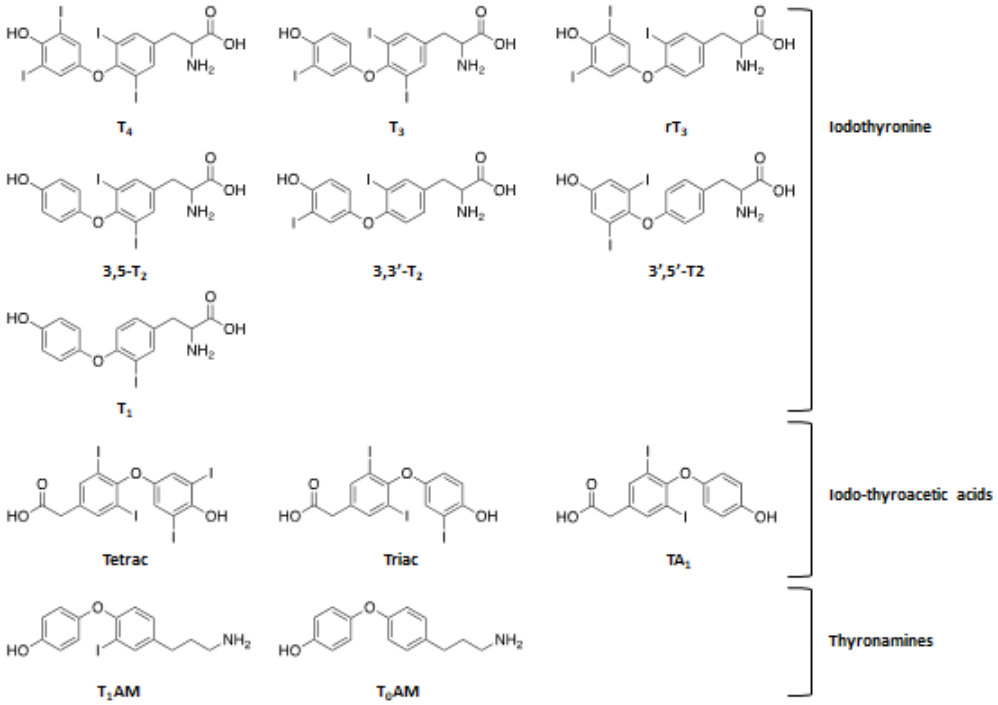

Figure 7: *Chemical structures of some of the main TH metabolites.*

1.5 DEHAL-1: structure, function and clinical implications

All the steps involved in the synthesis of TH, starting from I^- uptake from the bloodstream to the final synthesis and release of T3 and T4, have been already extensively illustrated (Par.1.1). TH are the only mammalian hormones containing a halogen in their structure, iodide, which is present at a very low concentration in the environment. Accordingly, thyroid functions depend on the availability of this halogen in the thyroid for the synthesis of TH. Dietary iodide is mainly ingested from sea salt, where it is present as NaI, milk, meat and fish, but daily I^- intake amounts alone are not sufficient to sustain the production of TH. For this purpose, the enzyme DEHAL-1 is responsible for the enzymatic deiodination of MIT and DIT generated in excess during TH synthesis [104]. MIT and DIT are released six-seven times more than TH during proteolysis of TG but they are inactive at thyroid level. The catalytic activity of DEHAL-1 produces the intrathyroidal I^- that, together with released Tyr molecules, can be recycled and reused for the synthesis of TH [105].

In the past, the existence of this deiodination activity has been already demonstrated even if the enzyme responsible for this reaction was still lacking. In 2003, Moreno *et al*., through the appli-

cation of the serial analysis of gene expression technique (SAGE) to thyroid tissue in combination with a computational method, identified the *DEHAL1* gene encoding for the protein responsible of the dehalogenation of TH. The gene was found to be mainly present in the thyroid gland and, with minor extent, in kidneys and liver [106]. Three isoforms of this enzyme were identified, namely DEHAL-1, DEHAL-1B and DEHAL-1C although only the first one showed dehalogenation activity, whereas the other two isoforms resulted inactive [107]. The structure of DEHAL-1 became clear after that the first crystal structure was reported by Thomas *et al*. in 2009 [108]. It has a molecular weight of 33 KDa and belongs to the NADH oxidase/flavin reductase superfamily, with a flavin mononucleotide (FMN) as a prosthetic group which is essential for its catalytic activity. T3 and T4 are neither deiodinated by DEHAL-1, nor able to bind to this enzyme, which does not contain the catalytic selenocysteine responsible for DIOs activity. DEHAL-1 is a transmembrane protein located at the apical membrane of the thyrocytes: it consists of a single transmembrane domain, a short carboxyl terminal domain and an amino terminal domain containing most of the nitro-reductase activity. The active site residues Glu-153, Tyr-157 and Lys-178 are responsible for the recognition of the zwitterionic forms of MIT and DIT as well as for the redox characteristics of the FMN. The alignment of the larger DIT over the isoalloxazine portion of the FMN is achieved through minor shifts of Leu-169, Thr-174 and Leu-172 (Figure 8).

The catalysed deiodination reaction occurs at the apical membrane in close proximity to TG and takes place during TG proteolysis both before and after its endocytosis into the thyrocytes [109]. The exact mechanism of the dehalogenation reaction is still under investigation, even if the previously proposed mechanism consisting in the simultaneous transfer of two electrons have been recently overcome by a single electron transfer mechanism. This putative process implicates a single electron transfer from the hydroquinone form of FMN and involves the biding of the phenolate form of iodotyrosines to DEHAL-1. The phenolate form enables the coordination with two hydrogen bond donors leading to the protonation of the halogen containing α-carbon. The protonated intermediate undergoes reductive dehalogenation through the electron donation from the hydroquinone form of FMN to form a transient semiquinone intermediate. The elimination of the halogen forms a stable phenoxy radical that can accept an electron from the semiquinone intermediate of FMN regenerating the oxidized form of FMN. The final product of the reaction is Tyr that, together with I^-, can be recycled to synthetize TH [110].

In vitro, DEHAL-1 activity showed to be dependent on NADPH levels and proportional to the enzyme concentration. The reported Km for the deiodination of MIT and DIT were $1.35x10^{-6}$ and $2.67x10^{-6}$, respectively, showing a higher affinity of DEHAL-1 for MIT respect to DIT. Fur-

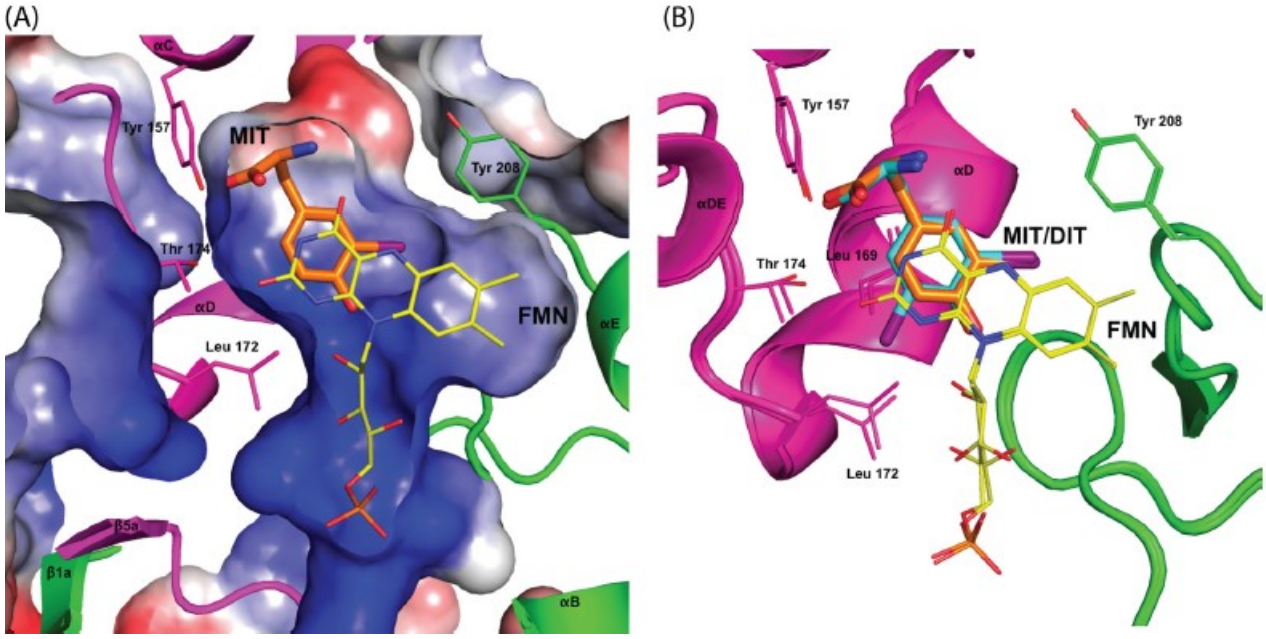

Figure 8: *Conformational changes of the DEHAL-1 active site to accommodate iodotyrosines. A. MIT-DEHAL-1 co-crystal structure showing the surface characteristics: blue indicates positive charges whereas red indicates negative charges; B. Conformational changes required for the alignment of DIT (cyan) to the active site of DEHAL-1 equally to MIT (orange) (Adapted from Thomas et al. [108]).*

thermore, *in vitro* and *in vivo* models showed that nitro aromatic compounds can be substrate of this nitro-reductase, revealing that the nitro-analogue 3,5-dinitro-L-Tyrosine (DNT) is a powerful DEHAL-1 inhibitor [40]. Moreover, DEHAL-1 is able to promote de-chlorination and de-bromination reactions of chloro-Tyr and bromo-Tyr, respectively.It was demonstrated that chloro- and bromo-Tyr were able to bind to the active site of DEHAL-1 with the same affinity as iodotyrosines, even if the dichlorination reaction resulted up to 20-fold slower. Conversely, DEHAL-1 was unable to defluorinated fluoro-Tyr that showed also a weaker binding ability to the enzyme respect to iodotyrosines [111]. Further studies reported the up-regulation of *Dehal1* mRNA in rats by TSH-stimulated cAMP and the down-regulation by acute and chronic administration of iodide. Moreover, the activity of DEHAL-1 was increased in hypothyroid rats [112].

In 2008, Moreno *et al*., reported the first human mutations of *DEHAL1*. Three different mutations were identified: two missense mutations (Arg101Trp and Ile116Thr) and one in-frame depletion of three base pair (Phe105-Ile106Leu). They were situated in the exon 2 of the gene encoding a FMN- binding site localized within the nitro-reductase domain of the protein. These mutations

drastically reduced the deiodination activity of DEHAL-1, leading to the release of MIT and DIT in plasma before their excretion through urine. The patients presented variable pathological phenotypes that can be related to the time of expression of the disease or to environmental factors, such as iodine intake. The four analysed patients came from unrelated consanguineous families, all of them had sever goitrous hypothyroidism and two of them suffered from intellectual deficits due to late diagnosis and treatment [113]. Failure of DEHAL-1 leads to an already known disease called iodotyrosine deiodinase deficiency (ITDD), which is characterized by hereditary hypothyroidism, goiter, psychomotor deficits and intellectual retardation when not properly treated in early stages of life. The identification of *DEHAL1* mutations opened novel opportunities to comprehend ITDD, considering the lack of expression at the beginning of life where TH play a crucial role in the development. In fact, two of the four patients analysed by Moreno *et al*., were subjected to the neonatal screening for hypothyroidism and, worryingly, resulted normal, meaning that the disease may not be present at the beginning of life [114]. An additional missense homozygous (HO) mutation (Ala220Thr) has been reported by Afink *et al*. in 2008 from another consanguineous family. The mutation, situated on the exon 4, resulted in an inactive DEHAL-1 activity *in vitro* showing both catalytic impairment and protein degradation. The two homozygous index patients (mother and daughter) showed hypothyroidism, goiter and, one of them, had mental retardation. High concentrations of MIT and DIT have been detected in urine from index patients. Interestingly, high levels of both iodotyrosines have been found also in one of the heterozygous sons at the age of 9, without any other symptoms. During the puberty (14 years old), clinical evidences of hypothyroidism with a massive goiter were discovered, demonstrating that phenotype can emerge over time [115]. Hitherto, the diagnosis of ITDD is not included in the neonatal screening programmes even if consequences in the neurodevelopment of untreated patients have been reported [114].

Recently, a novel *DEHAL1* homozygous mutation (p.A57SfsX62) was identified in the offspring of a consanguineous family from Lebanon. The mutation consisted in the insertion of an adenosine between the position 168 and 169 of the exon 1, causing a frameshift that led to an early-stop codon at the amino acid 62 of the protein. This novel mutation resulted in an almost completely truncated enzyme with the consequent delete of the functional nitro reductase domain and an impaired activity of DEHAL-1. The mutation was discovered in an index patient of 11 years old who consulted his physician because of a goitre developed in the last 3 weeks. Genetic investigation on *DEHAL1* gene mutations was extended to several member of the index patient's family (Figure 9). Genetic sequencing revealed the heterozygous condition of his parents and of the younger sister, whereas the older brother and the older sister resulted homozygous and the youngest sister was wild-type. At that time, phenotypic investigation identified goitre only in the index patient whereas none of

the other members showed any significant symptoms [116].

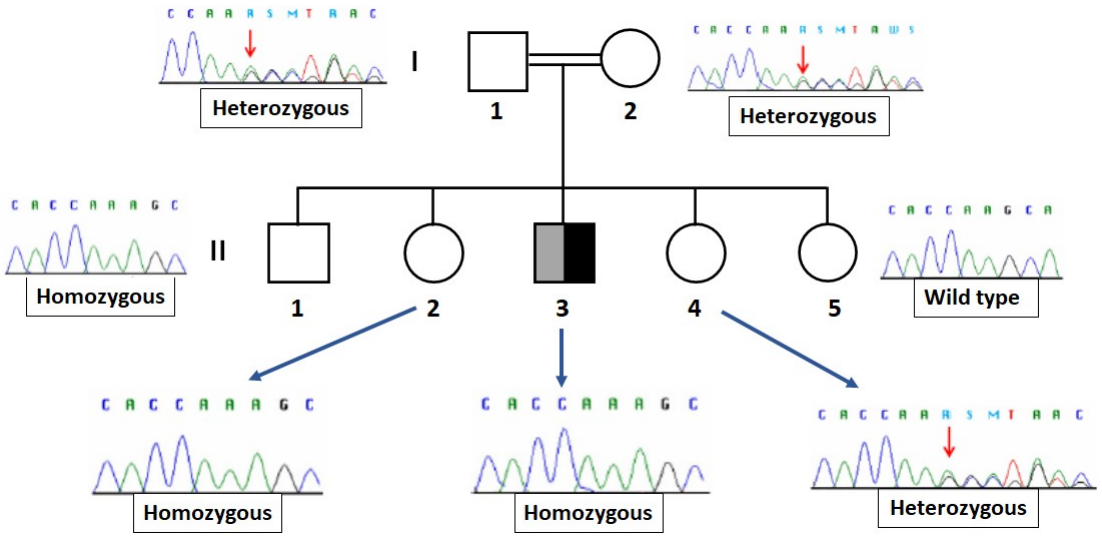

Figure 9: *DEHAL1 genotypic investigation of the index patient's family. The father (I-1) and the mother (I-2) resulted heterozygous. Among the siblings, two members (II-1 and II-2) other than the index patient, exhibited the homozygous mutation whereas the other sisters were heterozygous (II-4) and WT (II-5), respectively.*

2 High-performance liquid chromatography coupled to tandem mass spectrometry

2.1 General introduction to the technique

Liquid chromatography (LC) coupled to mass spectrometry (MS) is a powerful and versatile technique, widely used in analytical laboratories. In the chromatographic column the components of a mixture are separated based on their affinity for both the stationary phase and the mobile phase, and then ionized and detected by the coupled mass spectrometer.

With the term HPLC, we refer to a LC separation technique carried out at high operative pressure up to hundreds of bars or to thousands of bars in ultra-high pressure liquid chromatography (UHPLC) instruments. This separation technique relies on two main elements, namely stationary phase and mobile phase. The analytes are dissolved in an appropriate liquid mobile phase and pumped through the stationary phase, which is the hearth of the HPLC system, where the separation takes place. The stationary phase is constituted by a chromatographic column tightly packed with a solid

adsorbent material. Each compound interacts differently with both the adsorbent material present in the chromatographic column and the eluent. So that, it is eluted with a different time, known as retention time. In general, the higher is the affinity for the stationary phase (or the lower is that for the mobile phase), the later a compound is eluted from the column. Conversely, an analyte with a lower affinity for the stationary phase will be eluted with shorter retention times from the chromatographic column. The type of interactions depends on the compound chemical structure and, as a consequence, on its own physical-chemical characteristics, such as polarity, and on the characteristics of both stationary and mobile phases. Accordingly, stationary phase is accurately selected in order to achieve the best selectivity to separate the different components of a mixture [117]. Among the numerous stationary phases commercially available, a large part of the HPLC separations are carried out by the so-called reverse phase stationary phases. Differently from the classical type, known as normal phases, in which the stationary phase was made of silica or alumina, the surface of the silica particles is coated with siloxane together with various alkyl (C8 or C18) or aryl groups. The end part of the group is the one responsible for the interaction between stationary phase and the analytes. Reverse phase chromatography is indicated for the separation of a wide variety of different compounds and it is preferable considering that a large part of the suitable solvents are compatible with mass spectrometry, which is often coupled to HPLC.

Mobile phase is usually constituted of two solvents (typically an organic phase and an aqueous-phase) that are mixed together before being pumped into the system. Depending on the complexity of the mixture, the characteristics of analytes and the selected chromatographic column, the mobile phase is appropriately chosen. Its composition can be kept constant during the chromatographic analysis, creating what is known as isocratic condition. If the components of the mixture are poorly resolved under isocratic conditions, it is common to make use of a gradient condition where the composition of the mobile phase can vary during time. A typical gradient involves the gradual increase of the organic solvent with respect to water, up to reversing the relative concentration of the two solvents.

In the HPLC-MS technique, separated compounds eluted from the chromatographic column are detected with mass spectrometers. The coupling of the two techniques generates a sort of three dimensions chromatogram, known as ion chromatogram, which includes retention time, intensity, and mass spectra, where each peak identifies a different component of the mixture. In the last decades, mass spectrometry has rapidly evolved, and technological implementations led to the advent of more and more sensitive instruments.

The basic principle of MS is to generate ions from a compound by a proper ionization technique, to separate these ions according to their mass to charge ratio (m/z) and to detect them by their individual m/z and relative abundance. The basic scheme that all mass spectrometers follow implies an ion source, a mass analyser and a detector. The ions produced in the ionization step are directed to the high vacuum part of the mass spectrometer consisting of the mass analyser and the detector. Ions are accelerated through the mass analyser under magnetic and electric fields to be separated according to their m/z. Finally, the detector provides the ions abundances on the basis of their relative ion signal intensities, which are then plotted in order to obtain the mass spectra [118].

Ionization techniques are divided in two main groups, known as hard and soft techniques, that differs from each other based on the quantity of residual energy imparted to the molecules. Hard ionization techniques, such has electron ionization (EI), confer a high degree of fragmentation that is useful for structural identifications. Conversely, in soft ionization techniques the amount of residual energy is little and more suitable for the coupling with the HPLC technique. Examples of soft ionization techniques include, atmospheric-pressure photo ionization (APPI), atmospheric-pressure chemical ionization (APCI), electrospray (ESI), desorption electrospray ionization (DESI) and matrix-assisted laser desorption ionization (MALDI). Among all the ionization techniques, ESI interface was used for our experimental work and will be briefly described.

In the past, many of the existing ion sources were incompatible with a continuous liquid stream, such as the eluate of an HPLC. The situation changed when ESI was developed by Professor John Fenn in 1989 [119] and optimized by Prof. Andries Bruins. This ground-breaking interface, together with APCI, enabled the effective coupling of mass spectrometry with HPLC and, more recently, UHPLC. Considering the great impact that this invention had on protein and peptide biochemistry, Prof. Fenn was awarded with the Nobel Prize in chemistry, shared with Professor Koichi Tanaka, one of the developers of MALDI [120]. In ESI, the liquid is dispersed into a spray of electric charged droplets created through a metal capillary exposed to a high electric field (several kV). The solvent included into this highly charged spray is evaporated until the droplets become unstable, reaching what is known as Rayleigh limit. At this stage, droplets undergo deformation and the more the volume of the droplets decreases, the more the charge density at the droplet surface increases. When the electrostatic repulsions become stronger than the surface tension holding droplets together, they “explode” creating more stable and smaller droplets (Figure 10). The subsequent desolvation process causes the release of sample ions from the surface of the charged droplets that are then transported to the high vacuum part of the mass spectrometer to be analysed [121, 122].

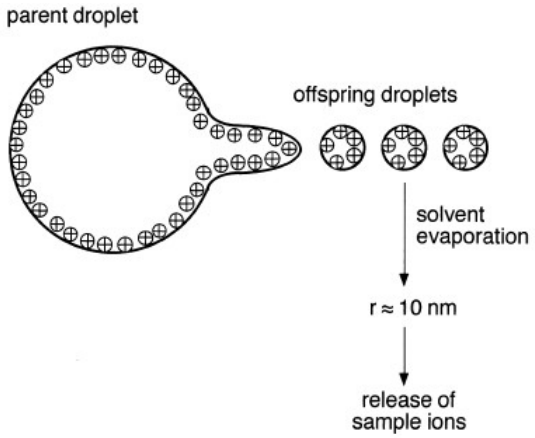

Figure 10: *Schematic representation of the charged droplet disintegration in ESI. The parent droplet releases several offspring droplets that undergo size reduction through solvent evaporation until they finally release the sample ions (Adapted from Bruins et al. [121]).*

Once generated, ions are transported into the mass analyser which is the hearth of the mass spectrometer, to be separated according to their m/z ratio. The aim of the analyser it to hold selected ions with specific masses and move them to the ion detector for counting. Different type of ions can be produced in the source: molecular ions with positive or negative charge (if the polarity in the ionization source is switched), components of the mobile phase and relative adducts from their combination with molecular ions, fragment ions if fragmentation is employed. There are more than a few types of analysers that can be employed for the separation of ions that use different methods to separate them according to their m/z: quadrupole mass analyser, ion trap mass analyser, time-of-flight mass analyser, Fourier transform analyser, magnetic and electromagnetic sector mass analyser and ion cyclotron resonance analyser [123]. For our experimental work we used quadrupole analysers and, more specifically, a triple quadrupoles analyser generating what is known as tandem mass spectrometry (MS/MS). The quadrupole consists of four parallel rods on which a direct and varying (radio frequency) voltages are applied. By varying the voltages during time, it is possible to scan across a range of m/z and select the transmission of specific ions through the axis of the rods creating the mass spectrum. MS/MS is a particular configuration that increases the specificity and sensitivity of the analysis, obtained by placing in series a collision cell (q2) between two quadrupoles (Q1 and Q3) (Figure 11). Fragmentation in the collision cell usually occurs with an inert collision gas, such as argon or nitrogen, and it known as collision-induced dissociation (CID).

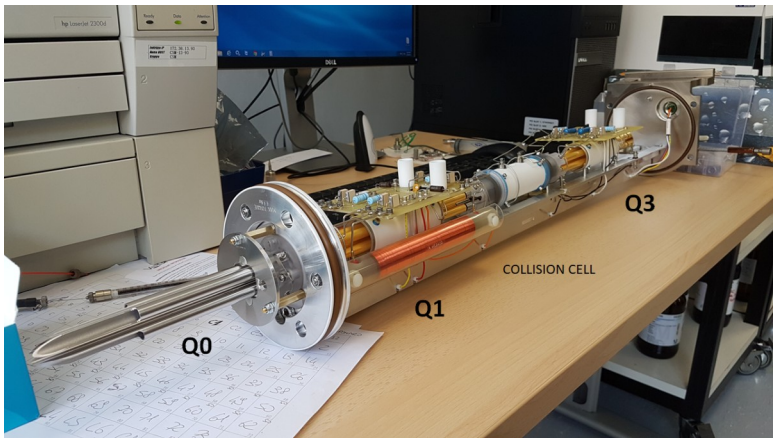

Figure 11: *Quadrupoles rail of the ABSciex API 4000 currently used in our laboratory. Q0 is a quadrupole used as ion guide and allows the transmission of ions to the triple quadrupoles system. Rods of Q1 and Q3 are made of porcelain covered with a thin gold layer and are placed on opposite sides of the collision cell where ions fragmentation occurs.*

Q1 and Q3 can operate in different scan modes that are illustrated in Figure 12: the "product ion scan" consists of the selection of an ion with a specific m/z in Q1 which is fragmented in q2. All the produced fragment ions are then scanned and detected using Q3; in the "precursor ion scan" Q3 focuses on a selected fragment ion produced in q2 whereas Q1 is scanning all the masses to identify the precursor ion; another common mode is the so-called "neutral loss scan" where both Q1 and Q3 scan all the ions included in a selected m/z range with a constant mass offset between the two quadrupoles. It detects all the fragmentations leading to a specific neutral fragment; the most used is the so-called Selected Reaction Monitoring (SRM), known also as Multiple Reaction Monitoring (MRM). In this mode there is no scan, since both Q1 and Q3 are focused on selected masses. Importantly, ions selected in Q1 are only detected if they produce a give fragment by a selected reaction.

After the mass analyser, ions are then detected and converted into a usable signal by a detector, a device able to transform incident ions into an electric current which is proportional to their abundance. Considering the minimal number of ions coming out from the mass analyser, an amplification of the signal is normally required in order to provide usable signals for data processing. At present, among the several available types of detectors, the electron multiplier is widely used. Briefly, positive and negative ions strike a conversion dynode triggering the emission of numerous secondary particles. These are then converted into electrons and amplified by a cascade effect to produce an electric current that is then processed by the data system and generate the mass spectra [117, 123].

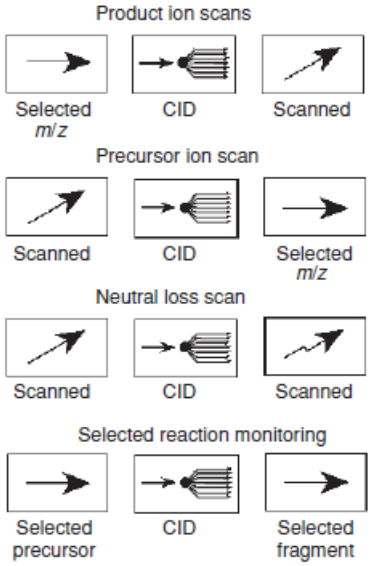

Figure 12: *Graphic description of the main scan modes used in MS/MS (adapted from DeHofmann et al. [117]).*

2.2 Importance of HPLC-MS-MS in TH analysis

TH are commonly assayed in serum and, sporadically, in plasma. As already discussed in Par 1.2, more than 90 % of circulating TH are bound to serum carrier protein. This implies the inaccessibility for tissues to the protein bound fraction which is considered inactive. In order to produce their genomic and non-genomic effects, TH dissociate from the carrier proteins and enter into the cells. However, a very small percentage (< 1%) is already present as free fraction (fT3 and fT4) which is directly accessible for peripheral tissues and, thus, considered as the biologically active fraction [124]. Considering the low concentration range of free TH (pM), it is evident how the quantification of the free fraction is more challenging respect to the total fraction (protein-bound + free). Moreover, the assay of the total fraction may not accurately reflect the thyroid status when protein concentrations or binding capacities are altered since this can affect the bound fraction without altering the levels of free TH. Because of these reasons, clinicians prefer to assess the levels of fT3 and fT4 on a routine basis [125].

The main methodologies that have been developed for the assay of serum fTH are divided in indirect and direct methods. The first one consisted in the mathematical estimation of the free fraction and involved the concomitant measurement of total TH (TTH) and TH binding proteins. The total concentration was then divided by TBG concentration or multiplied by an approximate value of

available TBG binding sites obtained through T3 and T4 uptake tests. This approach resulted in the evaluation of free T3 and T4 indexes that were usually requested by clinicians and were used in last decades [126]. Clearly, these indirect methods were dependent on the levels of TH binding proteins generating an underestimation or overestimation of TH levels. Moreover, the concentration of serum TH binding proteins can differ among the individuals and the method could only cover an ideal window of variability [127, 128].

Conversely, several direct immunoassays have been developed to assay fTH but require the physical separation of the free fraction prior the analysis. For this purpose, two main techniques are used to isolate the free fraction from the bound one, namely Equilibrium dialysis (ED) and Ultrafiltration (UF) that are not exempt from technical limitations. ED is subjected to dilution effects and to possible interference of the buffer components on the equilibrium between fTH and bound TH [129]. Conversely, UF is not affected by dilution effects and the yield of measured fTH is higher than ED [130]. However, some drawbacks were identified also for UF, such as membrane adsorption, protein leakage and susceptibility to pH and temperature [129].

Nowadays, most of the clinical parameters of the thyroid are determined using automated immunometric methods. In particular, highly sensitive (LOQ 0.7 – 0.07 pM, depending on the analytical system used) and automated immunoassays that generally use chemiluminescence detection, are currently employed as gold standards to detect serum fT3 and fT4 [131]. However, the accuracy of immunoassays can be affected by the variation of serum-binding protein concentration that can occur in case of pregnancy, genetic variations, medications that disrupt TH binding to serum proteins or certain medical conditions. Moreover, several studies have shown discrepancies in fTH results according to different immunoassays performed. This variability in fTH quantification may reflect differential assay susceptibility to the alteration in serum binding proteins [124]. Furthermore, the inverse log-linear relationship existing between fT4 (but also fT3) and TSH, poorly correlated when fTH were measured using immunoassays [132, 133].

Some of the above-mentioned weaknesses of the immunoassays, can be overcome with mass spectrometry. HPLC-MS-MS is a highly specific and sensitive technique able to detect low concentration hormones. A valuable advantage of HPLC-MS-MS is related to the use of stable isotope-labelled internal standards (ISs) analogues of TH that can be used to monitor the analytical process and compensate for analytical errors. Isotope dilution methods are widely applied in clinical MS and are used routinely for the clinical measurement of steroid hormones and Vitamin D [134, 135, 136]. HPLC-MS-MS is emerging as a powerful technique able to detect serum fTH

and TTH complementing the traditional immunoassay methods [124]. In addition, specific HPLC-MS-MS methods can be developed and used for the detection of TH in matrices other than serum, together with their metabolites, such as thyronamines, or their precursors, like MIT and DIT. However, a technical limitation of the HPLC-MS-MS technique is associated with the low throughput compared to automated high throughput immunoassay platforms.

Mass spectrometry was already involved in the quantification of serum TH since the seventies of the past century, and many GC-MS methods were developed [137, 138, 139, 140, 141, 142]. Despite their accuracy, these methods suffered from laborious sample clean-up and were time consuming and, so, they were abandoned with the advent of the ESI and APCI interfaces. This pioneering discovery made possible the effective coupling with HPLC systems and spurred the developing of highly sensitive and accurate HPLC-MS-MS methods able to detect serum T3 and T4 [142, 143].

Most of the developed HPLC-MS-MS methods are able to quantify the serum total fraction of TH. Due to the fact that most of circulating TH are bound to serum carrier protein, their detection usually requires a proteins precipitation step followed by sample purification. In fact, in the first proposed methods, acetone was used in the initial serum protein precipitation step, followed by a liquid-liquid extraction using ethyl acetate. These publications opened the way for the development of novel HPLC-MS-MS procedures and, in the following years, several research groups were able to reproduce these methods and introduced the more specific solid-phase extraction (SPE) to get a more selective isolation of TH [88, 144, 145, 146, 147, 148]. Over the years, many technological improvements made possible the development of increasingly sensitive mass spectrometry methods to assay TTH . Some researchers implemented the proposed methods and introduced, for example, an online extraction procedure or a derivatization step to convert TH in their corresponding butyl esters, in order to achieve the best sensitivity [149, 150, 151, 152, 153].

However, the interest of clinicians is focused on the assay of the free fraction of serum TH and as discussed above, most of the thyroid biochemical parameters are detected through high-throughput immunoassays. Despite the methodological difficulties of the assay that are mainly related to the serum fTH low concentrations, several HPLC-MS-MS methods have been developed and are emerging as gold standards. The first proposed methods employed the ED step in order to isolate the free fraction [154, 155, 156, 157]. These procedures were time consuming and required expensive devices so, in most cases, they have been replaced by the more rapid UF tandem mass spectrometry methods that resulted more sensitive and versatile [158, 159, 160, 161].

HPLC-MS-MS played a pivotal role for the quantification of TH together with most of their metabolites, i.e. rT3, 3,5-T2, 3,3'-T2 and thyronamines in serum and in other human matrices such as urine, saliva, breast milk and CSF. Moreover, mass spectrometry methods have been applied to assay the levels of these molecules in cell lysates and various tissues from animal models. The main advantage of this technique is the possibility to assay and quantify several molecules in a single run and with a small amount of sample, coupled to the possibility to transfer and validate the method to other matrices. An overview of the applications of HPLC-MS-MS for the quantification of TH and their metabolites have been extensive discussed in some reviews that were recently published [162, 163].

Part II

Aim of the project

Iodine deficiency has been associated with mental retardation due to the important role of TH in neuro development and it is one of the main causes of maternal hypothyroidism that can have critical consequences to the fetus, including irreversible brain damages [164]. Since the discovery of human *DEHAL1* mutations, the interest in ITDD increased and it is emerging as an important cause of iodine deficiency. The impairment of DEHAL-1 activity leads to the reduction of MIT and DIT deiodination and, therefore, to their excretion through urine [113].

Most of the absorbed iodine by the body is excreted through urine. However, urinary iodine does not offer direct information about thyroid status and cannot be used for the diagnosis and treatment of individuals [165]. Specifically, a prompt detection of ITDD before the onset of its symptoms and the related hypothyroidism condition would be extremely useful in order to begin the treatment. Consequently, a method able to quantify MIT and DIT in urine would be beneficial in clinics in order to screen for ITDD. Clinical application of HPLC-MS-MS is increasing due to its selectivity, specificity and robustness and, at present, only one method able to quantify MIT and DIT in urine was published by Afink *et al*. in 2008 [115].

The aim of my project was to develop and validate an accurate HPLC-MS-MS method able to detect MIT and DIT in urine and plasma, together with TH in the latter case.

Our method was applied for the analysis of plasma and urine collected from the novel *Dehal1* KO mouse to complete its biochemical characterization and to identify the timing of hypothyroidism onset. The generation of this *Dehal1* KO mouse is the first mammalian model of defective iodine recycling caused by DEHAL-1 deletion. Furthermore, we tested the ability of our HPLC-MS-MS method to detect MIT and DIT levels in urine collected from a reported DEHAL-1 deficient family. The detection of urinary MIT and DIT as pre-clinical biomarkers of hypothyroidism in ITDD could be potentially interesting as part of the new-born screening.

Part III

Materials and Methods

3 Chemicals and reagents

Acetonitrile (ACN), methanol (MeOH), ultra-pure water, methylene chloride (CH_2Cl_2), 2-propanol or iso-propanol, acetone, hydrochloric acid (HCl), glacial acetic acid (AcOH), ammonium hydroxide (NH_4OH), potassium acetate ~98% (AcK) and 3.0 N HCl in n-butanol were purchased from Sigma-Aldrich (Saint Louis, MO, USA) and were all LCMS grade. Formic acid (FA) was provided by Biosolve-chemicals (Dieuze, France) and was LCMS grade.

Analytical standards of MIT, DIT, T3, T4 and stable isotope labelled $^{13}C_6$-T3 and $^{13}C_6$-T4 were also supplied by Sigma-Aldrich. Stable isotope labelled $^{13}C_9$-MIT and $^{13}C_9$-DIT were kindly provided by Prof. Alireza Mani (University College of London, UK), since they were not commercially available.

4 *Dehal1* knock-out mice samples collection

Dehal1 KO mice were generated by Prof. José Carlos Moreno and the colony was maintained by Dr. Cristian González Guerrero in the Thyroid Research Laboratory (University Hospital La Paz, Madrid, Spain). The generation of the first *Dehal1* KO goes beyond my PhD project and will be concisely described. The production of the KO mice was supported by the KO mouse project repository (KOMP). A specific targeting vector containing lacZ was used as reporter gene, whereas Neomycin as selection gene and inserted by homologous recombination between exon 1 and exon 2 of the *Dehal1* wild-type (WT) allele. As a result, DEHAL-1 protein's expression resulted truncated.

C57BL/6J mice have been cross bred to obtain homozygous (HO) mutants. To confirm the generation of the KO and to differentiate the three possible genotypes WT, heterozygous (HT) and HO, genotype analysis was performed via RT-PCR on mouse tail genomic DNA. Electrophoresis gels, molecular studies and immunohistochemistry analysis confirmed the absence of the DEHAL-1 protein in KO conversely to WT in which the protein was constitutively expressed [166].

4.1 Iodine deficiency model

Female 5-6 months old C57BL/6J *Dehal1* WT and KO mice were subjected to specific free iodine pellet diet (TD180914, Envigo, Indianapolis, IN, USA) containing 0.25 µg of I/g. Mice were divided in three groups corresponding to three different iodine containing diets. Drinking water iodine content was 1400 µg of I/L, 200 µg of I/L and without any iodine supplementation in normal iodine diet (NID), low iodine diet (LID) and very low iodine diet (VLID), respectively. Mice daily iodine intake corresponded to 5.6 µg of I, 1 µg of I and 0.25 µg of I for NID, LID and VLID, respectively. Urine was accumulated through a special hydrophobic sand (Sodispan Research, Madrid, Spain) and collected at day 0, 12, 28. Samples were centrifuged at 1500 rpm for 5 min and supernatants were collected before storage. At the same time points, blood was collected from the submandibular vein and plasma was obtained by centrifugation at 2000 rpm for 10 min. Samples were stored at -80°C until the extraction and then, analysed through HPLC-MS-MS.

4.2 Natural early-life model

Pregnant female C57BL/6J *Dehal1* WT and KO mice were subjected to a NID. Plasma was collected as mentioned before from 10 days old mice (pups) and 1 month old (juvenile) together with urine in the latter case. Samples were stored at -80°C until extraction to be submitted to HPLC-MS-MS analysis.

5 Human DEHAL-1 family samples collection

Urine was collected from members of the Lebanon consanguineous family that has been introduced at the end of Par. 1.5 and from four control patients. The HO frameshift mutation in DEHAL-1 (p.A57SfsX62) in the index patient (II-3) was reported (Figure 13), whereas his father (I-1, 40 years old) and his mother (I-2, 36 years old) resulted HT. Among the siblings, samples were collected from his older brother (II-2, 19 years old) and older sister (II-2, 17 years old) that were HO and from one of his younger sisters that resulted HT (II-4, 13 years old). We were not able to collect urine form the youngest WT sister (II-5, 5 years old). Urine was stored at -80°C until being submitted to HPLC-MS-MS extraction and analysis to quantify levels of MIT and DIT.

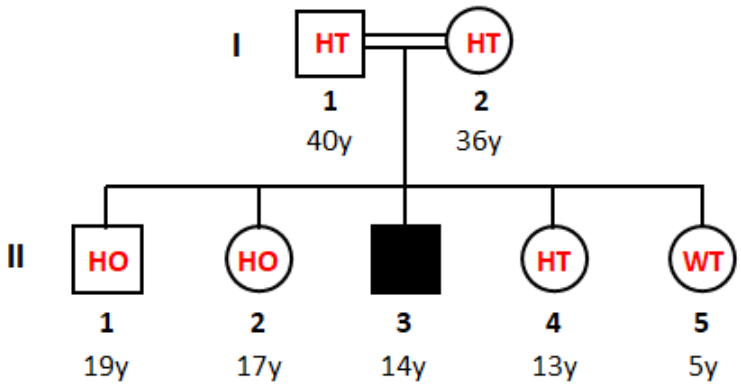

Figure 13: *Pedigree of the Lebanon consanguineous family. The black square indicates the index patient who was the only member of the family showing goitre and hypothyroidism. HO, HT and WT refers to the DEHAL-1 p.A57SfsX62 mutation.*

6 HPLC-MS-MS conditions

6.1 Instrumental layout and operative conditions

Instrument layout was made up of an Agilent (Santa Clara, CA, USA) 1290 UHPLC system, comprehending a binary pump, a column oven set to 20°C and a thermostated autosampler, coupled to an ABSciex (Concord, Ontario, Canada) QTRAP 6500+ triple quadrupole mass spectrometer, equipped with an IonDrive™ Turbo V source. The integrated switching valve was used to discard both head and tail of the HPLC runs. Chromatographic separations were carried out using a 110 Å, 2x50 mm, 3µm particle size, Gemini C18 column (Phenomenex, Torrance, CA), protected by a C18 Security Guard Cartridge. System control, data acquisition and analyses were performed using an ABSciex Analyst® version 1.7 software.

1µL of each sample were injected to the UHPLC system and the chromatographic separations were carried out with a flow rate of 400 µl min^{-1} using MeOH/ACN (20/80 by volume) added with 0.1% FA as solvent A and water containing 0.1% FA as solvent B. Mobile phases gradient conditions are showed in Table 1. Mass spectrometry selected reaction monitoring (SRM) method operated in positive ion mode. For each compound, after the optimization of declustering potential (DP), collision energy (CE) and collision exit potential (CxP), three transitions were considered in the analysis. Based on the highest signal/noise ratios, one of them was used as quantifier (Q) and the other two as qualifiers (q) (Table 2).

HPLC binary pump			
Total time (min)	Flow rate (µL/min)	Solvent A (%)	Solvent B (%)
0.1	400	5	95
3.0	400	5	95
8.5	400	65	35
9.0	400	100	0
11.0	400	100	0
11.5	400	5	95
14.0	400	5	95

Table 1: *HPLC pumps gradient. Solvent A: MeOH/ACN (20/80 by volume) + 0.1% FA; Solvent B: water + 0.1% FA.*

MS parameters					
Analyte	SRM transitions (Da)		DP (V)	CE (V)	CxP (V)
MIT-C_4H_9	363.9	135.0 (q)	65.0	44.0	10.3
	363.9	261.8 (Q)	65.0	25.0	14.1
	363.9	291.0 (q)	65.0	22.0	16.1
$^{13}C_9$-MIT-C_4H_9	373.1	143.2 (q)	95.0	44.0	10.3
	373.1	270.1 (Q)	95.0	25.0	14.1
	373.1	300.2 (q)	95.0	22.0	16.1
DIT-C_4H_9	489.9	260.9 (q)	55.0	51.2	7.7
	489.9	290.0 (q)	55.0	36.0	8.3
	489.9	387.9 (Q)	55.0	31.4	11.0
$^{13}C_9$-DIT-C_4H_9	498.8	268.8 (q)	64.0	48.0	5.9
	498.8	298.9 (q)	64.0	35.0	7.1
	498.8	395.8 (Q)	64.0	28.0	9.5
T3-C_4H_9	707.9	479.1 (q)	84.0	53.5	12.3
	707.9	605.9 (Q)	84.0	38.2	17.2
	707.9	651.9 (q)	84.0	26.7	19.0
$^{13}C_6$-T3-C_4H_9	713.9	485.1 (q)	84.0	53.5	12.3
	713.9	611.9 (Q)	84.0	38.2	17.2
	713.9	657.9 (q)	84.0	26.7	19.0
T4-C_4H_9	833.9	605.0 (q)	82.0	60.7	15.5
	833.9	731.9 (Q)	82.0	43.4	19.5
	833.9	777.9 (q)	82.0	29.5	22.6
$^{13}C_6$-T4-C_4H_9	839.9	611.0 (q)	82.0	60.7	15.5
	839.9	737.9 (Q)	82.0	43.4	19.5
	839.9	783.8 (q)	82.0	29.5	22.6

Table 2: *MS operative parameters. The quantifier transitions (Q) have been accurately selected and used for the quantification of the analytes, while the other two (q) have been used only for a qualitative purpose.*

Additional operative parameters were set as follow: Collision gas (CAD) nitrogen; operative pression with CAD gas, 5.6 mPa; Curtain gas (CUR), 20 arbitrary units; Gas source 1 (GS1), 70 arbitrary units; Gas Source 2 (GS2), 55 arbitrary units; ion spray voltage (ISV), 5.25 kV; Source temperature (TEM), 650 °C; Entrance potential (EP), 10 V; Focusing lens 1 (IQ1), -10.1 V.

6.2 Samples extraction

Previously published methods [88, 151] have been modified in order to allow the simultaneous quantification of MIT, DIT, T3, T4. Briefly, 100 µL of urine/plasma were placed in a 2mL Eppendorf tube with an appropriate amount (5.4 pmol $^{13}C_9$-MIT, 4.0 pmol $^{13}C_9$-DIT, 2.8 pmol $^{13}C_6$-T3, 2.4 pmol $^{13}C_6$-T4) of stable isotope-labelled ISs. Samples were vortexed, equilibrated for 30 min at room temperature, 300µL of cold-acetone were added and samples were kept 30 min at 4°C to allow proteins precipitation. As mentioned in the previous section, the majority of HPLC-MS-MS methods are able to quantify the total fraction of TH. The use of organic solvents reduces the hydration of the proteins and leads to their aggregation and precipitation. Consequently, the TH-bound fraction is released and, together with the free TH fraction, can be detected as total TH. After centrifugation at 22780 x g for 10 min, the supernatants were transferred to a new 2 mL Eppendorf tube and dried at 40 °C under a gentle stream of nitrogen. The concentrated samples were derivatized adding 200 µL of 3.0 N HCl in n-butanol and incubated for 45 min at 60 °C. This derivatization step allows the formation of the corresponding butyl esters of tyrosines, TH and their ISs. Afterwards, samples were dried again as mentioned above and then, reconstituted with 500 µL of 0,1 M AcK (pH=4) prior to loading onto Agilent (Santa Clara, CA, USA) Bond-Elut Certify 130 mg SPE cartridges. Before loading the samples, cartridges have been previously conditioned by consecutive wetting with 2 mL of CH_2Cl_2/isopropanol (75/25 by volume), 2 mL of methanol and 2 mL of 0,1 M AcK (pH = 4). After consecutive washes with 3.5 mL of water, 2 mL of 0.1 M HCl, 7 mL of MeOH and 3.5 mL of CH_2Cl_2/isopropanol (75/25 by volume), samples were eluted with 2 mL of CH_2Cl_2/isopropanol/NH_4OH (70/26.5/3.5 by volume). Eluates were dried under nitrogen, reconstituted with 100 µL of ACN / 0.1 M HCl (50/50 by volume) and injected into the HPLC-MS-MS system. Stock solutions of MIT, DIT, T3 and T4 were prepared at 1 µg/mL concentration in methanol. Calibration curves were daily prepared by serial dilution with methanol at a concentration ranging from 0.1 to 100 ng/mL and derivatized with samples.

6.3 Method validation

The analytical method was validated with reference to selectivity, linearity, sensitivity, accuracy, precision, recovery and matrix effect [167, 168]. Selectivity was determined in order to evaluate the ability of the method to differentiate all the molecules of interest and their relative ISs from all the other components and interferents present in the samples. It was determined by repeated injections of the analytes into the system and their retention times were monitored. Linearity was evaluated within the calibration curve range built as described in the previous paragraph and instrumental sensitivity was assessed by evaluating limits of detection (LOD) and limit of quantification (LOQ) of the analytes. Using a specific tool of ABSciex Analyst® software, the concentrations providing a S/N ratio close to 3 and 10, were assumed as LOD and LOQ, respectively. Accuracy (%), calculated by the formula [(measured concentration/nominal concentration spiked) x 100], was measured on human plasma and urine which were spiked with 3 different concentration levels of analytes (2.5 ng/mL, 10 ng/mL and 50 ng/mL) and subtracted from the endogenous concentration. Intra-day and inter-day precision were evaluated and expressed as relative SD (RSD %): intra-day was precision was assessed injecting 5 replicates of the above mentioned concentration levels within the same run; inter-day precision was evaluated injecting the same 3 concentration levels on 5 consecutive days. Recovery and matrix effect were assessed in human plasma and urine. Recovery (%) was evaluated by comparing the peak areas of the IS added before and after the extraction procedure (4 replicates per matrix). It was calculated by the formula [(peak area of IS added before the extraction/peak area of IS added after the extraction) x 100]. The estimation of matrix effect (%) was evaluated comparing the peak area of the IS added to water (A) and matrix (B) both previously submitted to the extraction process (4 replicates per matrix). Matrix effect was then calculated by the formula [(B/A) x 100].

As part of the validation process, the ability of our method to quantify MIT, DIT and TH in mice urine and plasma from a pilot experiment has been tested. Female 5-6 months C57BL/6J *Dehal1* WT and KO mice were subjected to a NID. Urine and blood were collected at day 28 as mentioned in Par. 4 and submitted to HPLC-MS-MS analysis.

7 Study approval

All animal studies followed guidelines outlined by the European Community and Animal Research Ethical Committee guidelines, following approval by the Autonomous University of Madrid Animal Research Ethical Committee (CEI, Madrid, Spain). All the research involving human beings

has been reviewed and approved by the human research ethics committee.

8 Statistical analysis

Data analysis of the method validation was performed using Microsoft Excel (Microsoft Office, Redmond, WA) and results are reported as mean ± standard deviation (SD). GraphPad Prism 8® (GraphPad software, San Diego, CA) was used for data processing and statistical analysis of mice and human experiments. Results are reported as mean ± standard error mean (SEM). In the pilot experiment and natural early life model statistical difference between WT and KO groups was evaluated by non-parametric Mann-Withney's test for two independent samples. In the iodine deficiency model statistical significance between WT and KO was evaluated using multiple t-test corrected for multiple comparison using the Holm-Sidak method.

Part IV

Results and Discussion

9 Method development and validation

HPLC-MS-MS methods have been widely used for the analysis of TH in different matrices, such as cell lysates, culture mediums, a wide variety of mice tissues and human plasma and serum. However, none of them allowed the simultaneous quantification of TH with their precursors MIT and DIT. The importance of the detection of iodotyrosines in plasma and, especially in urine, have already been extensively discussed. So far, only one HPLC-MS-MS able to detect MIT and DIT in urine has been proposed by Afink *et al*. in 2008. This method had an important role in the study of DEHAL-1 deficiency and it was used for the quantification of MIT and DIT in human urine from consanguineous families [115, 169]. As proposed in our method, samples were derivatized using a butanolic HCl solution, in order to form the corresponding butyl esters. This derivatization process has been frequently applied in TH analysis and the advantage is related the increase of the signal-to-noise ratio (S/N) due to a higher in-source ionization efficiency and to a better interaction of some of the derivatized analytes with the C18 reverse-phase column. The yield of the esterification reaction has to be considered and optimized during the validation of the method [151, 163]. The main disadvantage of the method proposed by Afink *et al*. is related to the IS used for the quantification of MIT and DIT. Considering that stable isotope labelled IS for MIT and DIT were not commercially available, 3-chloro-L-tyrosine was used for both molecules. In our method, we were able to use stable isotope-labelled MIT and DIT molecules kindly provided by Prof. Alireza Mani (Division of Medicine, University College of London, UK), namely $^{13}C_9$-MIT and $^{13}C_9$-DIT. The availability of a proper IS for each compound that co-elute with the analytes, is beneficial in terms of normalization of compound signals and it is advantageous in overcoming the matrix effect. In urine, only MIT and DIT were considered in the analysis considering the low amounts of T3 and T4 in this fluid. Moreover, TH are excreted through urine as conjugated metabolites that increase their water solubility and, consequently, are not susceptible to the derivatization reaction proposed in our method [84]. The aim of the method validation is to demonstrate the reliability of a specific method for the determination of an analyte concentration in a biological matrix, that in our case corresponded to plasma and urine [168].

The developed HPLC-MS-MS method was consistent with the analytical purpose and showed excellent selectivity for all the analytes of interest and their corresponding IS. (Figure 14).

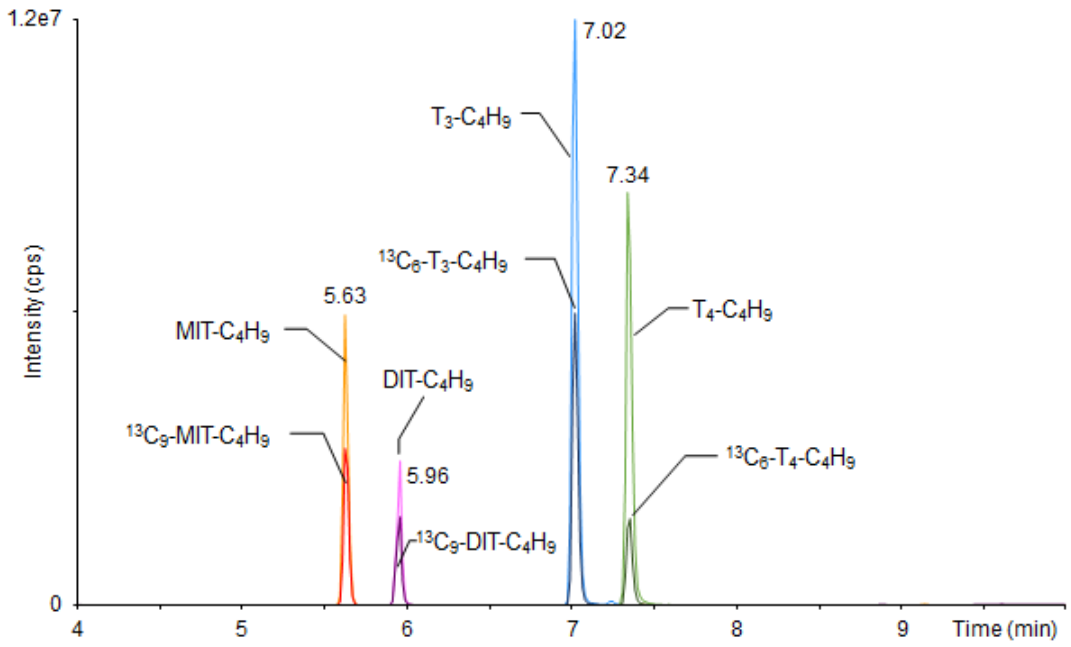

Figure 14: *HPLC-MS-MS chromatogram of a standard solution containing butylated MIT, DIT, T3 and T4 and their relative ISs. The peaks of each compound have been obtained by summing the three MRM traces monitored during the analysis. The x-axis is zoomed in the time range of 4-10 min which corresponds to the scheduled ionization time range.*

Experimental results reported in Table 3 revealed that chromatographic retention times were stable and calibration curves, with a general equation y = mx + q obtained with a weighed linear regression (1/x), showed a linearity always higher than 0.998 in the tested concentration range.

Analyte	Retention time (min)	Slope (m)	Intercept (q)	Correlation (r^2)
MIT-C_4H_9	5.63 ± 0.01	0.0521 ± 0.0129	0.0050 ± 0.0018	0.9991 ± 0.0006
DIT-C_4H_9	5.96 ± 0.01	0.1388 ± 0.0252	0.0089 ± 0.0048	0.9991 ± 0.0006
T3-C_4H_9	7.02 ± 0.01	0.0342 ± 0.0190	0.0023 ± 0.0021	0.9989 ± 0.0009
T4-C_4H_9	7.34 ± 0.01	0.0245 ± 0.0020	-0.0001 ± 0.0013	0.9988 ± 0.0006

Table 3: *Some of the method performances showing retention times and calibration curves equations. Values are expressed as mean ± SD.*

Instrumental sensitivity was evaluated in standard solution and LOD and LOQ values are reported in Table 4. and matched with the method requirements. Moreover, considering MIT and DIT

results, our method showed a sensitivity even higher than the one reported by Afink *et al*., where LOD values in water were 0.20 nM for both MIT and DIT [115].

	LOD		LOQ	
Analyte	ng/mL	nM	ng/mL	nM
MIT-C_4H_9	0.05	0.14	0.10	0.28
DIT-C_4H_9	0.02	0.04	0.10	0.20
T3-C_4H_9	0.01	0.01	0.02	0.03
T4-C_4H_9	0.01	0.02	0.05	0.06

Table 4: *Instrumental LOD and LOQ of iodotyrosines and TH.*

Accuracy represents an important validation parameter showing how the assayed concentration of the analyte reflects its nominal concentration. It was evaluated by spiking known amounts of the four analytes to urine or plasma samples at three concentration levels, namely 2.5, 10, 50 ng/mL, subtracted of the endogenous amounts. Results reported in Table 5 revealed satisfactory results for all the analytes both in plasma and urine, except for an outlier result detected for DIT in urine at the highest tested concentration.

	Plasma			Urine		
Analyte	Spiked (ng/mL)	Assayed (ng/mL)	Accuracy (%)	Spiked (ng/mL)	Assayed (ng/mL)	Accuracy (%)
MIT-C_4H_9	2.50	2.77	110.8	2.50	2.18	87.3
	10.00	10.18	101.8	10.00	10.83	108.3
	50.00	49.16	98.3	50.00	53.93	107.8
DIT-C_4H_9	2.50	2.30	92.2	2.50	2.65	106.1
	10.00	9.81	98.1	10.00	11.19	111.9
	50.00	48.56	97.1	50.00	62.50	124.9
T3-C_4H_9	2.50	2.28	91.2			
	10.00	9.78	97.8			
	50.00	52.28	104.6			
T4-C_4H_9	2.50	2.60	104.0			
	10.00	10.35	103.5			
	50.00	46.50	93.0			

Table 5: *Accuracy results for plasma and urine. The assayed concentration was obtained by subtracting the endogenous concentration to the spiked concentration.*

Intra-day and inter-day precision (also expressed as the coefficient of variation) describe the closeness of repeated individual measures of analyte. They are expressed as RSD (%) and should not exceed 15%. Our experimental results showed good intra-day and inter-day precision for all the

injected concentration levels with values in the range of 3.68-11.30 % and 2.85-10.36 %, respectively. Recovery estimates the efficacy of the extraction process and was calculated by comparing the peak area of the IS spiked before and after the SPE process. An ideal analytical process should possess a recovery of 100%, which is very unlikely to obtain. Due to the endogenous presence of the analytes of interest, the use of spiked IS has been chosen in order to evaluate the recovery, allowing a better analysis of the entire process. The recovery rates are reported in Table 6 and showed satisfying results for all the analytes, with the lowest values observed for T3 and T4 in plasma, ranging at 76.2 % and 63.8 %, respectively. High recovery rates are beneficial since they have an impact on the method sensitivity. Conversely, recovery does not influence the accuracy of the method because of the presence of ISs that, as already stated, behave as the corresponding analytes. Moreover, the absence of isotopic effects when using ^{13}C-labelled ISs is advantageous compared to deuterated-labelled ISs.

The so-called matrix effect is due to co-eluting and undetected matrix components present in the sample which can significantly affect the ionization efficiency. This well-known effect using ESI technique, can induce ion suppression or enhancement, two phenomena that can affect the reproducibility, the accuracy and the robustness of the assay [167]. Therefore, the evaluation of matrix effect is crucial in developing an HPLC-MS-MS method and as stated before, an advantage of our method was the availability of a proper IS for all the analyte of interest. IS coelute with their related compounds and are affected by an equal matrix effect. For these reasons, matrix effect was evaluated by comparing the IS peak area spiked after the extraction process with the IS peak area of the neat solution, in which the same amount of IS was spiked in 100 µL ACN / 0.1M HCl. Experimental results reported in Table 6 displayed a general trend of ion suppression except for MIT and DIT that presented a slight ion enhancement, especially in urine.

	Plasma		Urine	
Analyte	Recovery (%)	Matrix effect (%)	Recovery (%)	Matrix effect (%)
$^{13}C_9$-MIT-C_4H_9	84.4	78.9	84.6	105.0
$^{13}C_9$-DIT-C_4H_9	94.1	112.2	87.2	107.3
$^{13}C_6$-T3-C_4H_9	76.2	99.6		
$^{13}C_6$-T4-C_4H_9	63.8	81.0		

Table 6: *Recovery and matrix effect for plasma and urine. Matrix effect values > 100% indicate ionization enhancement; Matrix effect values < 100% indicate ionization suppression.*

In order to complete the validation process, the method has been tested and used for the quantification of MIT, DIT and TH in plasma and urine from a pilot experiment. The method showed to be a

powerful analytical technique to detect all the analytes of interest (Figure 15).

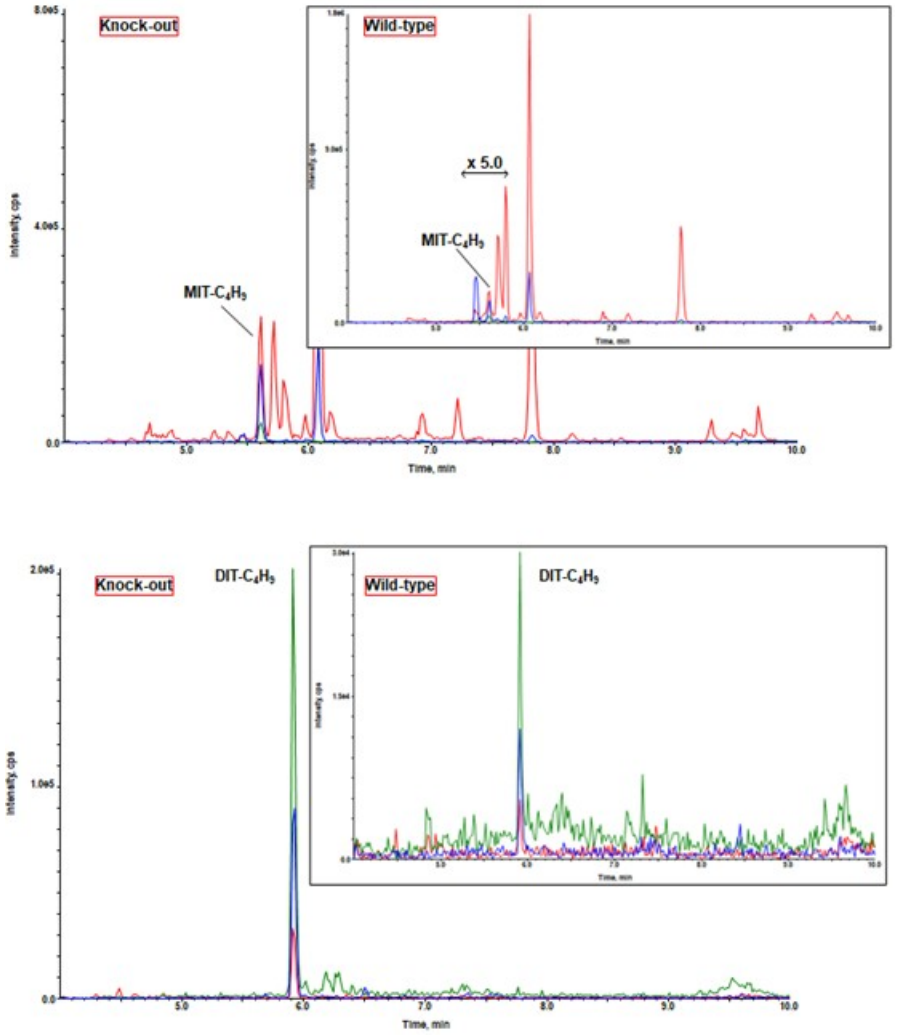

Figure 15: *Representative chromatograms of plasmatic MIT and DIT from WT and KO mice. Even from a visual point of view, the massive difference between WT and KO could be appreciated. The peak under the label "x 5.0" was amplified by a factor 5 to make it more clearly visible.*

Results showed in Figure 16 and 17 are consistent with a defected DEHAL-1 activity, showing a general increase of MIT and DIT levels in KO mice plasma and urine respect to WT. In KO samples, MIT and DIT could not be deiodinated by DEHAL-1, resulting in an increase of their levels in plasma together with the increased excretion of both molecules through urine [113]. On the other hand, under this normal iodine dietary intake condition, the thyroid was still able to synthetize TH and, as a consequence, their levels did not show any statical significance between WT and KO.

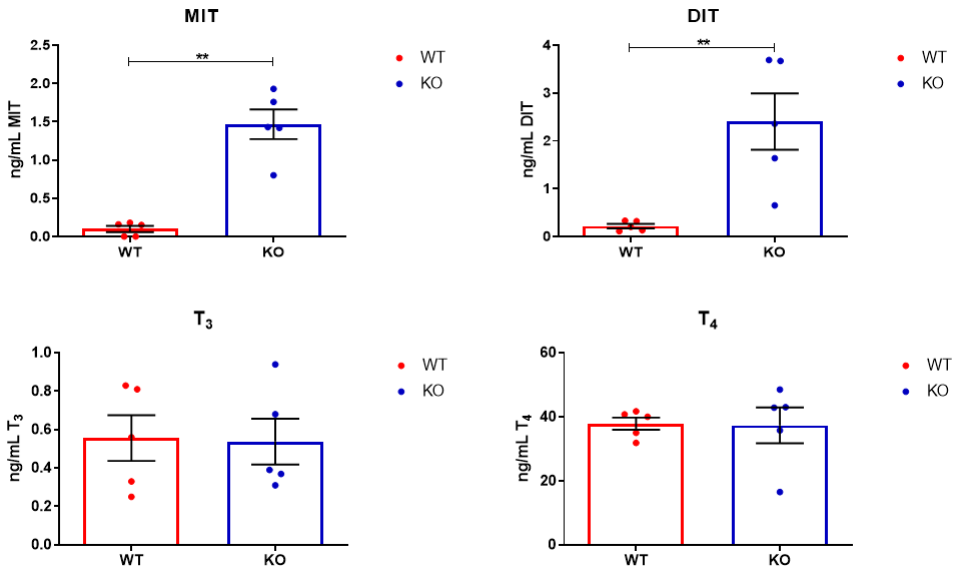

Figure 16: *Scatter plot with bar graphs showing levels of MIT, DIT and TH in mice plasma samples from the pilot experiment. (n=5; ** vs WT p< 0.01).*

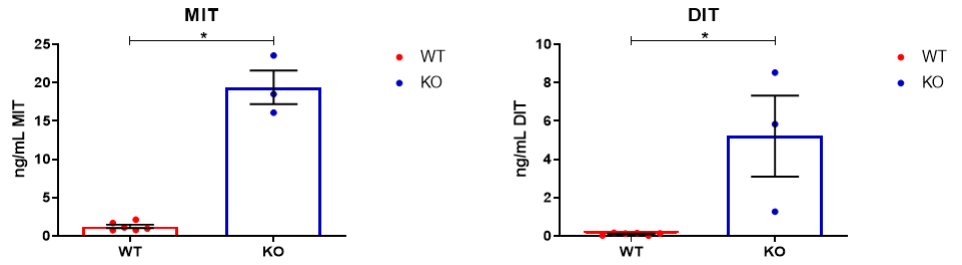

Figure 17: *Scatter plot with bar graphs of mice urine levels of MIT, DIT from the pilot experiment. (n=5 for WT and n=3 for KO; * p< 0.05).*

Altogether, the experimental data reported above provided satisfactory results that fitted with the analytical requests. However, the proposed method was not exempted from minor imperfections that have to be considered. The main flaw was related to the processing time. The method was time consuming, mainly caused by the derivatization step coupled to a double evaporation of the samples. The derivatization step resulted to be critical for the retention of MIT into the SPE cartridge in order to be selectively isolated and extracted. Several attempts trying to avoid the derivatization step have been done resulting in the loss of most of the analytes, especially MIT, during the SPE and during the HPLC chromatographic separation. Moreover, a better chromatographic separation into the C18 HPLC column was achieved after the derivatization. (data not shown). However, as

reported above, the method provided good results in terms of robustness, accuracy and selectivity and was successfully employed for our research projects.

10 *Dehal1* knock-out mice

10.1 Iodine deficiency model

The iodine deficiency model was designed to investigate how the DEHAL-1 deficiency is influenced by the iodine availability and how this could affect TH synthesis and trigger the establishment of an hypothyroidism condition.

At day 0, under NID (5.6 µg of I / day), the results showed a general trend that was similar to the ones found in the pilot experiment, with higher levels of MIT and DIT detected in KO respect to WT in both plasma and urine samples (Figure 18).

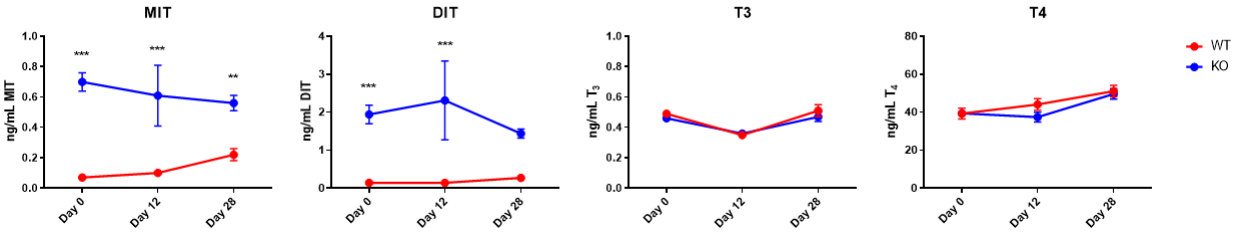

Figure 18: *XY graphs showing levels of MIT, DIT and TH in mice plasma subjected for 28 days to NID. Mean ± SEM (For WT n=23 at day 0, n=7 at day 12 and n=6 at day 28 whereas for KO n=23 at day 0, n=8 at day 12 and n=7 at day 28). ** vs WT (p< 0.01), *** vs WT (p<0.001).*

Within day 0 and day 28, MIT and DIT levels in WT were close to the LOQ of the method oscillating in the range of 0.10±0.01 to 0.22±0.04 ng/mL and 0.14±0.02 to 0.27±0.03 ng/mL, respectively. In plasma from KO mice MIT and DIT levels significantly increased up to 0.70±0.06 and 2.31±1.04 ng/mL, respectively and persisted higher than WT till day 28. T3 and T4 concentrations in WT fluctuated from 0.49± 0.02 to 0.51± 0.04 ng/mL and from 39.30± 2.85 to 51.08± 3.09 ng/mL, respectively. Interestingly, levels of both T3 and T4 in KO did not significantly differ from WT and remained stable even at day 28, meaning that mice were still euthyroid. Considering the importance of DEHAL-1 to supply the thyroid with most of the required iodine to synthetize TH, our results showed that in KO mice the thyroid was able to overcome this DEHAL-1 deficiency and to maintain the levels of T3 and T4 within the normal range by using iodine from the diet.

As expected, we observed a massive increase of MIT and DIT levels in urine from KO mice that persisted till day 28 (Figure 19).

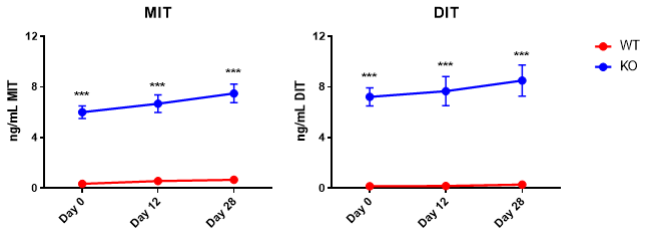

Figure 19: *XY graphs showing urine levels of MIT and DIT after 28 days under NID. Mean ± SEM (For WT n=23 at day 0, n=7 at day 12 and n=7 at day 28 whereas for KO n=24 at day 0, n=8 at day 12 and n=8 at day 28). *** vs WT (p<0.001).*

Under NID, WT urinary levels of MIT and DIT were close to the instrumental LOQ and varied between 0.34±0.11 and 0.66±0.11 ng/mL for the first one, and between 0.15±0.02 and 0.28±0.03 ng/mL for the latter one. In KO samples, we detected MIT concentration 11.3 to 17.6 folds higher than WT (6.00±0.50 ng/mL to 7.48±0.73 ng/mL). The effect was even more prominent for DIT whose levels in KO samples increased up to 30.4 to 48.1 folds (7.21±0.72 ng/mL to 8.50±1.23 ng/mL).

The results reported in Figure 20, illustrate levels of MIT, DIT, T3 and T4 in plasma when daily iodine intake was decreased down to 1 µg, namely LID. Elevated levels of MIT and DIT in KO respect to WT persisted till day 12 ranging at 0.47±0.07 and 1.66±0.38 ng/mL, respectively, even if a decreasing trend during time was detected. At day 28, the concentrations of both analytes in KO were still higher than WT but in the same concentration ranges, specifically 0.34±0.08 ng/mL and 0.49±0.09 ng/mL for MIT and DIT, respectively. On the other hand, TH concentrations detected in plasma were normal at all the time points and no significant differences between WT and KO were detected.

The same trend was also found in urine in which the concentration of both MIT and DIT in KO decreased during time even if still significantly higher than WT (Figure 21). At day 28, due to stringent iodine conditions, we detected 2.61±0.49 ng/mL of MIT and 2.70±0.37 ng/mL of DIT in KO samples, almost 3 times lower respect to the ones detected at day 0, ranging at 6.00±0.50 ng/mL and 7.21±0.72 ng/mL, respectively. Strikingly, these results revealed the ability of the thyroid to maintain TH levels within the normal range in this DEHAL-1 deficient model even if the amount of dietary iodine intake was lowered to 1 µg /day. Although the levels of MIT and DIT decreased, the thyroid could still be able to use the low amount of iodine from the diet or could activate

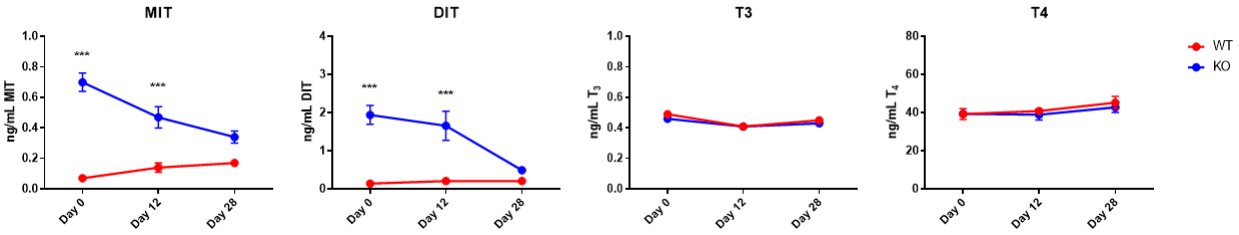

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Figure 20: *XY graphs showing levels of MIT, DIT and TH in mice plasma subjected for 28 days to LID. Mean ± SEM (For both WT and KO n=23 at day 0, n=8 at day 12 and n=8 at day 28). *** vs WT (p<0.001).*

some alternative iodine recycling mechanisms to synthetize TH. We could also hypothesize that the decreased levels of excreted MIT and DIT in both plasma and urine, could represent a putative mechanism to save iodine into the thyroid to be used for the synthesis of TH.

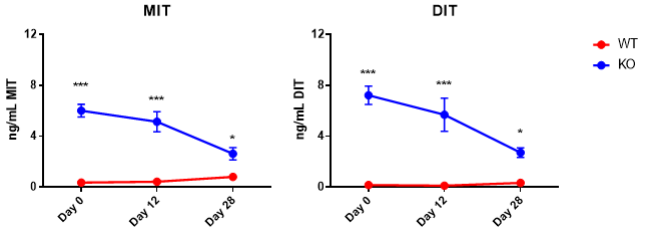

Figure 21: *XY graphs showing urine levels of MIT and DIT after 28 days under LID. Mean ± SEM (For WT n=23 at day 0, n=8 at day 12 and n=8 at day 28 whereas for KO n=24 at day 0, n=8 at day 12 and n=8 at day 28). * vs WT (p<0.05), *** vs WT (p<0.001).*

In figure 22 plasmatic concentrations of iodotyrosines and TH under VLID are reported. Under this very severe iodine deficient diet, both MIT and DIT levels in KO decreased during time reaching very low concentrations at day 28. In fact, at this last time point, the concentration of MIT was 0.27±0.03 ng/mL whereas DIT was 0.25±0.03 ng/mL and resulted in the same ranges of WT samples that were 0.19±0.02 ng/mL and 0.16±0.02 ng/mL, respectively.

This extremely low iodine diet condition did not affect plasma TH levels in WT that continued to be normal during time even if a decreasing trend for T4 could be observed. Remarkably, in KO samples T4 decreased during time and, already at day 12, reached a concentration of 17.24±0.82 ng/mL that was significantly lower respect to WT ranging 33.94±1.63 ng/mL. At day 28, T4 concentration in WT was 28.35±1.82 ng/mL whereas in KO it became critically low reaching 5.61±1.11 ng/mL, seven times lower than the one found at day 0. Interestingly, we detected an increase of KO levels of T3 at day 12 that reached a concentration of 0.87±0.09 ng/mL. However, T3 dramatically decreased to a final concentration of 0.14±0.02 ng/mL at day 28. We hypothesized that the increased

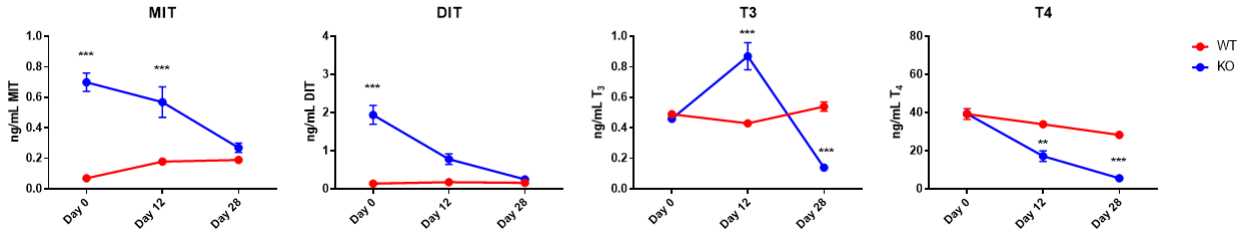

Figure 22: *XY graphs showing levels of MIT, DIT and TH in mice plasma subjected for 28 days to VLID. Mean ± (For WT n=23 at day 0, n=8 at day 12 and n=8 at day 28 whereas for KO n=23 at day 0, n=7 at day 12 and n=8 at day 28). ** vs WT (p< 0.01), *** vs WT (p<0.001).*

concentration of T3 detected in KO at day 12 could be explained as a mechanism of the thyroid to overcome the extremely low iodine conditions and synthetize the active hormone T3. In conclusion, after 28 days under this extreme low iodine diet, KO mice became hypothyroid and showed very low levels of both T3 and T4.

As illustrated in Figure 23, levels of MIT and DIT in urine from KO mice were significantly higher than WT, with the latter close to the instrumental LOQ. We detected a reduction of the concentrations of both analytes during time, with MIT that decreased from 6.00±0.50 ng/mL to 3.39±0.25 ng/mL whereas DIT diminished from 7.21±0.72 ng/mL to 1.44±0.24 ng/mL after 28 days.

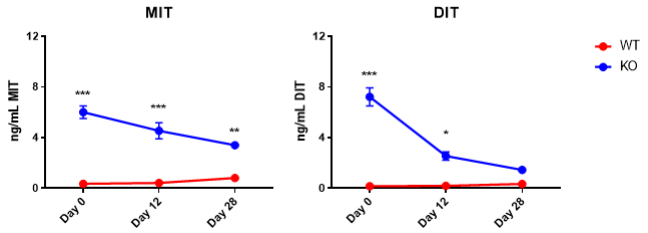

Figure 23: *XY graphs showing urine levels of MIT and DIT after 28 days under VLID. Mean ± SEM (For WT n=23 at day 0, n=8 at day 12 and n=8 at day 28 whereas for KO n=24 at day 0, n=8 at day 12 and n=8 at day 28). * vs WT (p<0.05), ** vs WT (p< 0.01), *** vs WT (p<0.001).*

Taken together, these results revealed that hypothyroidism condition was triggered by the progressive iodine deficiency clearly evident under VLID and that MIT and DIT loss preceded the establishment of this pathological condition.

10.2 Natural early-life model

TH are essential during pregnancy and have a fundamental role in fetal development. Untreated maternal hypothyroidism is correlated with higher risk of adverse outcomes during pregnancy, especially for the child [170]. Furthermore, congenital hypothyroidism is one of the main causes of mental impairment and growth retardation in infants. It is included in the new-born screening of many countries and involves the measurement of TSH and T4 from blood spots. The new-born screening is considered as one of the major achievements in the preventive medicine and consents the screening for a wide variety of congenital pathologies that can be treated but are not clinically evident shortly after birth [171]. Hitherto, the diagnosis of ITDD is not included in the neonatal screening programmes even if neurodevelopmental consequences of untreated patients have been reported [114]. As showed above by our experiments, adult *Dehal1* KO mice showed significant loss of MIT and DIT in urine under basal condition. The aim of this second experimental model was to investigate if a similar trend could be detected during mice development and how early ITDD can be diagnosed.

Experimental results reported in figure 24 confirmed that MIT and DIT levels were already elevated at day 10 in KO pups respect to WT.

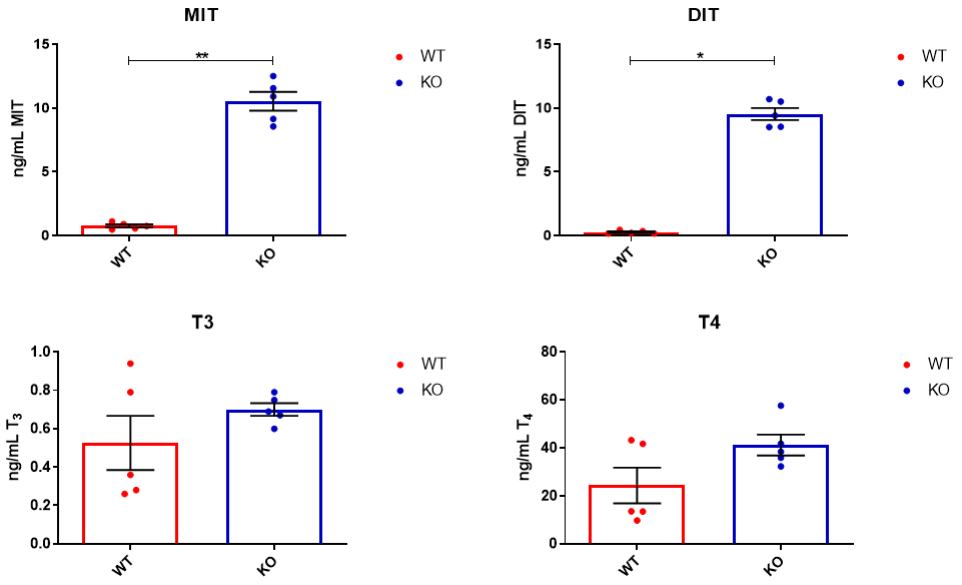

Figure 24: *Scatter blot with bar graphs illustrating the plasmatic levels of MIT, DIT and TH in 10 days mice. Mean ± SEM (n=5 for both WT and KO). * vs WT (p<0.05), ** vs WT (p< 0.01).*

In fact, a significant increase in plasmatic levels of MIT and DIT were detected in KO samples and

reached 10.53±0.74 ng/mL and 9.53±0.47 ng/mL, respectively. Remarkably, T3 and T4 were still within the normal range and did not differ between WT and KO, meaning that at this stage of life mice were still euthyroid, even if the plasmatic loss of MIT and DIT caused by ITDD was already evident.

The same trend was observed in plasma from juvenile mice and the reported concentrations in Figure 25 confirmed an increase of MIT and DIT in KO whereas in WT they were close to the instrumental LOQ. The levels of MIT and DIT reached 0.59±15 ng/mL and 1.40±0.59, respectively, but were an order of magnitude lower than the ones detected in 10 days mice. At this stage of life, mice were still euthyroid and levels of both T3 and T4 did not differ between WT and KO and fluctuated within the normal range.

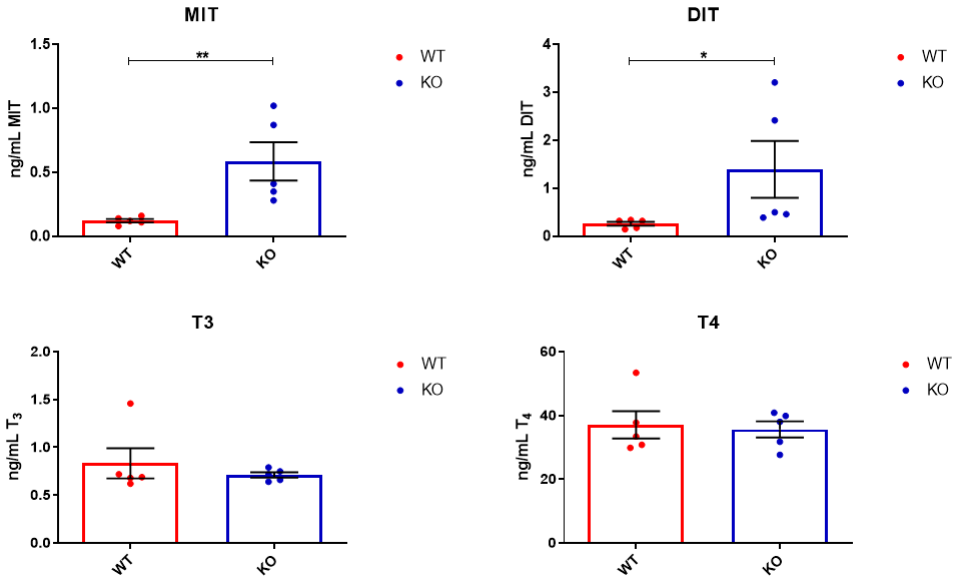

Figure 25: *Scatter blot with bar graphs showing the concentration of MIT, DIT and TH found in plasma from juveniles. Data are expressed as Mean ± SEM (n=5 for both WT and KO). * vs WT (p<0.05), ** vs WT (p< 0.01).*

We detected a massive loss of urinary MIT and DIT in KO juvenile mice (Figure 26). As previously discussed, an hallmark of ITDD is the excretion of MIT and DIT through urine and our findings matched the expected results. In fact, while the WT urinary concentrations of both analytes were close to the LOQ, we detected 10.01±1.68 ng/mL of MIT and 2.30±0.86 ng/mL of DIT in KO samples, significantly higher than WT.

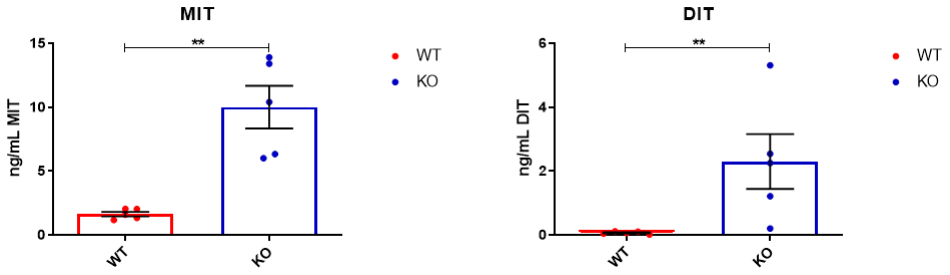

Figure 26: *Scatter blot with bar showing the concentration of MIT and DIT in juvenile urine. Data are expressed as Mean ± SEM. (n=5 for both WT and KO). ** vs WT (p< 0.01).*

In summary, from the beginning of life, even under a proper iodine availability, heavy amounts of plasmatic and urinary MIT and DIT were already present in *Dehal1* KO mice. These results supported the possibility to use the detection of excreted MIT and DIT in urine as a biomarker for ITDD, even in the first stages of life.

11 Human DEHAL-1 family

In addition to the characterization of the *Dehal1* KO mice models, we had the opportunity to apply and to test our HPLC-MS-MS to human samples in order to get some preliminary results on the quantification of MIT and DIT in urine. Information about the index patient, his family and the collection of urine samples have been introduced in the materials and methods section (Par. 5). Before illustrating the results of MIT and DIT levels, a brief summary of the clinical case examined by Dr. Esther Schulz (Altona Children's Hospital, Hamburg, Germany), is here described.

The 11 years old index patient consulted his physician because of a large goitre developed in the last 3 weeks. The initial laboratory results reported in Table 7 confirmed an hypothyroidism condition, with elevated levels of TSH and reduced concentration of fT4 and fT3, with the latter still within the normal range.

TSH (0.30 - 4.50 mU/I)	fT3 (0.78 – 1.54 ng/dL)	fT4 (2.20 – 4.70 ng/dL)
>150	0.20	2.40

Table 7: *Thyroid status parameters of the index patient before starting the replacement therapy with L-T4.*

From the clinical point of view, permanent hypothyroidism, mainly caused by Hashimoto's thyroiditis, requires lifelong Levothyroxine (L-T4) treatment whereas reversible hypothyroidism demands only short-term treatment with L-T4. The therapeutic goal of the treatment is the stabilization of TSH levels within the normal range and particular care must be taken in choosing the right dose of L-T4 considering, for example, age, other pathological conditions or concomitant drug therapies and pregnancy. The patient must be monitored after 6-12 months in order to evaluate clinical and biochemical parameters. The follow-up of the patient is important to understand the adherence and the efficacy of the replacement therapy and, if necessary, to plan a dose correction [172].

For these reasons, the patient started the treatment with L-T4 75μg/day and after 2 weeks a shrinking of the goitre was reported. The patient was monitored for the following years although non-compliance with the therapy was reported. Interestingly, inconsistent clinical results were reported in October 2013 when, after 3 to 5 months without any L-T4 intake, TSH and TH levels were normal, as illustrated in Table 8, and only a mild elevated goitre size was diagnosed.

TSH (0.53 – 3.59 μIU/mL)	fT3 (2.30 – 5.00 pg/mL)	fT4 (0.90 – 1.60 ng/dL)
1.53	4.80	0.98

Table 8: *Thyroid status parameters of the index patient after 3 to 5 months without replacement therapy with L-T4.*

In June 2014, after the reliable administration of 37.5 μg/day of L-T4 for 6 months and unintentionally reduced fish consumption, the index patient showed an increased goitre, elevated TSH concentrations, reduced fT3 and fT4 levels and a massive increase of TG concentration (Table 9).

TSH (0.53 – 3.59 μIU/mL)	fT3 (2.30 – 5.00 pg/mL)	fT4 (0.90 – 1.60 ng/dL)	TG (3.50 – 77.00 ng/mL)
100.00	1.10	< 0.40	8828.00

Table 9: *Thyroid status parameters of the index patient after 6 months of reliable good adherence to the replacement therapy with L-T4.*

The dose of L-T4 was then increased up to 150 μg/day and, in the last follow up in June 2015, the volume of the goitre was shrunk and levels of TSH and TH were normal, as illustrated in Table 10. Considering all these data and the clinical course, an ITDD has been suggested specifically looking at the presence of some of the distinctive features of ITDD, such as: long lasting normal TH levels during therapy pauses; development of hypothyroidism and goitre during Levothyroxine

TSH (0.53 – 3.59 μIU/mL)	fT3 (2.30 – 5.00 pg/mL)	fT4 (0.90 – 1.60 ng/dL)
0.52	4.58	1.24

Table 10: *Levels of TSH and TH of the index patient found in the last follow up in June 2015.*

replacement therapy and reduced dietary iodine intake; increase and reduction of goitre volume within short periods of time[116] . Consequently, genetic investigation on *DEHAL1* gene has been extended to several member of the index patient and has been already reported in Par. 1.5.

With regard to urinary MIT and DIT levels, we were able to assay both molecules and a representative chromatogram is presented in Figure 27.

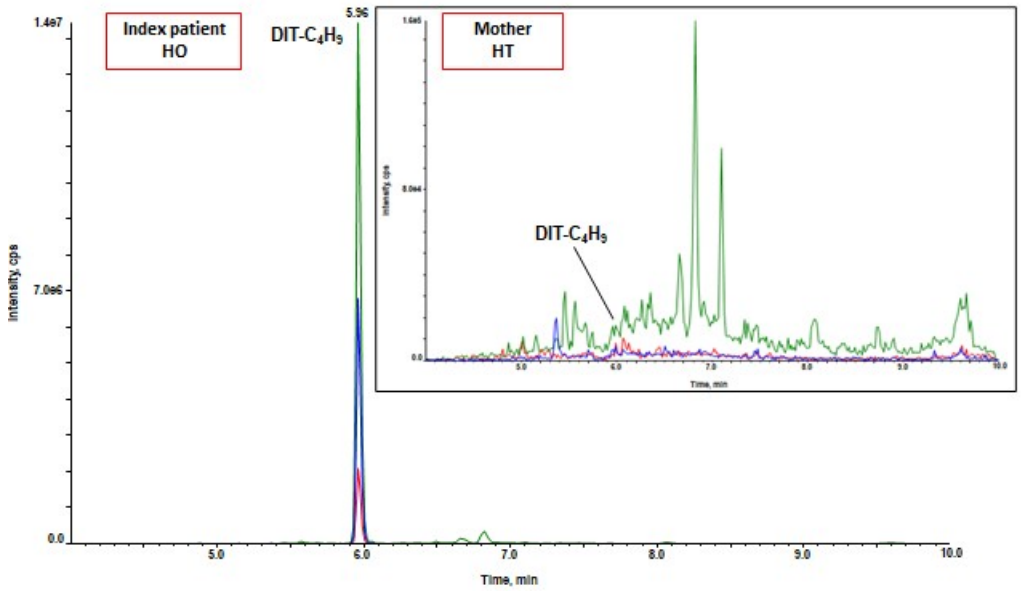

Figure 27: *Representative chromatograms of DIT assay in human urine. A massive increase of urinary DIT levels was detected in the index patient (HO), whereas DIT was almost undetectable in the mother (HT).*

Even from a visual examination of the proposed chromatograms, it can be appreciated the enormous increase of DIT levels in the index patient. In fact, as illustrated in Figure 28, a massive increase of both MIT and DIT levels were detected in the index patient (II-3) respect to control with a concentration of 316.00 ng/mL and 129.00 ng/mL, respectively, confirming the diagnosis of ITDD for the index patient.

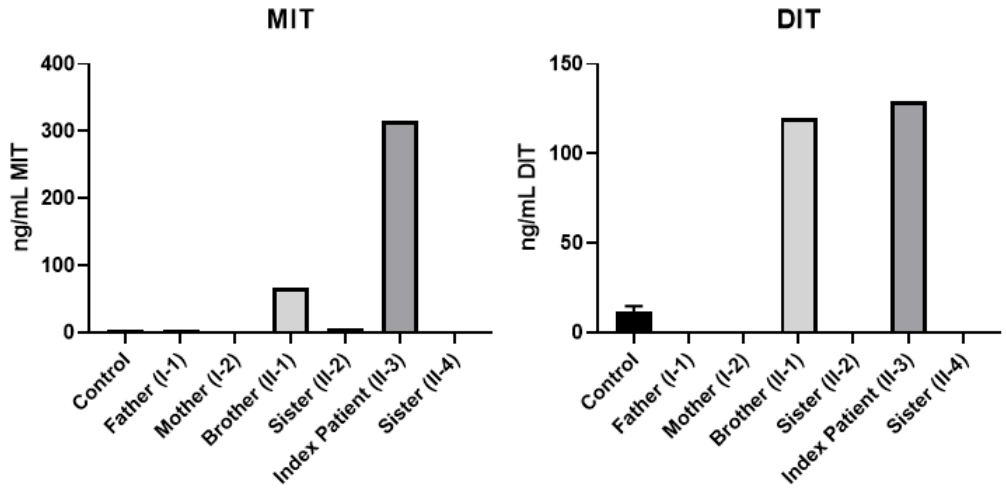

Figure 28: *Column graphs with MIT and DIT levels found in urine from human patients. The results from the 4 samples selected as controls are expressed as Mean±SEM.*

At this time, the 15 years old boy presented goitre and hypothyroidism conditions described by elevated levels of TSH and fT4 and a massive increase of TG (Table 11).

At his last clinical follow-up, the index patient had a good regression of the goitre after levothyroxine treatment and dietary iodine supplementation and any thyroidectomy surgery was reported.

Both father (I-1) and mother (I-2) were known to be HT and the detected levels of MIT and DIT were close to the instrumental LOQ. As illustrated by Table 11, no hypothyroidism conditions were reported and values of TSH, fT4 and TG looked normal.

Remarkably, we detected elevated urinary MIT and DIT levels in the brother (II-1), ranging 66.10 ng/mL and 120 ng/mL, respectively, confirmed by the detected homozygous genotype (Figure 9). These results highlighted elevated concentrations of both analytes in urine when the patient did not suffer from any other clinically evident thyroid disorders, yet. In fact, the reported values for TSH, fT4 and TG in Table 11 were in the normal and no goitre was detected, confirming the euthyroid conditions of the patient.

Patient	Phenotype	Genotype	TSH (0.55–4.68) mUI/L	fT4 (0.89-1.76) ng/dL	TG (0.83-84.00) ng/mL
Father (I-1)	Euthyroid	HT	1.17	1.28	10.20
Mother (I-2)	Euthyroid	HT	0.59	1.08	22.40
Brother (II-1)	Euthyroid	HO	0.63	1.22	18.10
Sister (II-2)	Euthyroid	HO	1.02	1.17	18.60
Index (II-3)	Hypo, Goitre	HO	14.11	0.42	5583.00
Sister (II-4)	Euthyroid	HT	0.98	0.96	14.20

Table 11: *Clinical picture of the index patient's family at the time of urine samples collection.*

At the age of 21, the brother consulted the physician after starting to develop symptoms of hypothyroidism in the last 4 weeks, such as fatigue and difficulties to swallow. After clinical evaluation, the patient showed goitre and the results of TSH, TG and fT4 analysis are reported in Table 12. The elevated levels of TSH and TG together with low levels of fT4 confirmed the diagnosis of hypothyroidism.

TSH (0.53 – 3.59 μIU/mL)	fT3 (2.30 – 5.00 pg/mL)	fT4 (0.90 – 1.60 ng/dL)	TG (3.50 – 77.00 ng/mL)
76.14	4.94	0.45	3649.00

Table 12: *Thyroid parameters of the oldest brother of the index patient after clinical follow-up.*

The patient reported no significant differences respect to the first control in which, as already reported, TH levels were in the normal range and the patient was still euthyroid. He only referred a psychological pressure for his upcoming marriage but no variations in terms of diet and, more specifically, in terms of dietary iodine intake. These results confirmed the importance of the detection of MIT and DIT as a pre-clinical biomarker of hypothyroidism.

Clinical results in Table 11 revealed an euthyroid clinical picture for the older sister (II-2) of the index patient, with no evidence of goitre and thyroid parameters within the normal intervals. MIT and DIT levels in urine detected with our HPLC-MS-MS did not significantly differ from control patients, even if genetic analysis detected an homozygous mutation of *DEHAL1*. At the time of the last follow-up, the patient did not present any hypothyroidism symptoms, she was married and has had a child. She reported an ordinary pregnancy without any levothyroxine treatment and informed the physician on the health conditions of the 3 years old child confirming the absence of any signs of hypothyroidism.

Part V

Conclusions

During my PhD I had the chance deeply understand most of the principles of mass spectrometry with a focus on HPLC-MS-MS. I was involved in the difficult analytical task of TH assay and I could explore the potential and the limitations of HPLC-MS-MS technique applied to TH analysis.

I had the opportunity to spend 3 years at the University of Pisa, Italy, in the research group of Prof. Riccardo Zucchi. I was supervised by Prof. Alessandro Saba who is the technical head of the Centre of Mass Spectrometry at the Santa Chiara University Hospital of Pisa. My main project was related to the set-up, development and validation of an efficient HPLC-MS-MS method for the quantification of MIT and DIT in mice urine and plasma, together with TH in the latter case.

We had the opportunity to collaborate with Prof. José Carlos Moreno (University Hospital La Paz, Madrid, Spain), an expert in the field of TH and iodine deficiencies, who discovered the first human *DEHAL1* mutation. The novel *Dehal1* KO mice was developed and produced in his laboratory and we contributed to the biochemical characterization of this mouse model with our HPLC-MS-MS method.

We were able to detect the increase of plasmatic and urinary MIT and DIT levels in *Dehal1* KO mice. These results were in accordance with the impaired activity of the DEHAL-1 enzyme that was no longer able to recycle iodine through the deiodination of MIT and DIT molecules. Moreover, our results showed the ability of the thyroid to overcome this iodine deficiency and maintain the levels of TH within the normal range. However, when the iodine diet content was extremely low (VLID), the availability of this essential element was too limited and, as a consequence, the synthesis of TH was impaired, and mice became hypothyroid. This confirms the importance of dietary iodine intake that becomes crucial in the presence of ITDD.

Another important result was obtained by looking at the early stages of life. We detected increased plasmatic levels of MIT and DIT in *Dehal1* KO mice already at 10 days of age (pups), that persisted till day 30 (juvenile) where high concentration of both molecules was also found in urine. However, at the beginning of life, mice were still euthyroid, since levels of detected T3 and T4 were normal.

Finally, we could apply our HPLC-MS-MS for the quantification of MIT and DIT in urine from

the members of family with a confirmed DEHAL-1 deficiency. We detected a massive urinary loss of MIT and DIT in the index patient possessing a DEHAL-1 homozygous mutation. This was in accordance with the hallmark of the ITDD and was a further confirmation of the disease. Strikingly, we were able to detect the increase of urinary MIT and DIT levels in a member of the family that had an homozygous DEHAL-1 mutation without showing any symptoms at the time of sample collection. However, some years later, the patient developed a massive goitre and typical symptoms of hypothyroidism.

Altogether, these results supported the hypothesis that MIT and DIT could be considered as pre-clinical biomarkers of hypothyroidism in DEHAL-1 deficient mice and humans. Considered the importance of TH in the development, the detection of iodotyrosines could be included as part of the human new-born screening for hypothyroidism. Our proposed HPLC-MS-MS method fulfil the analytical requirements and could put the basis for the development of a suitable and rapid method to slowly enter into the clinical laboratory.

Part VI

Pubblications

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