# The thyroid... in the periphery!

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# The thyroid... in the periphery!

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# Presentation

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Head of IBSA Foundation for Scientific Research

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The Forum "The thyroid... in the periphery!" took place in Naples, Italy, on the 15th of April, 2016, at the Federico II University. The purpose of this Forum, organized by IBSA Foundation with the support of Domenico Salvatore, Associate Professor of Endocrinology, Department of Molecular and Clinical Endocrinology, Federico II University, was to discuss about thyroid not usually looking at its diseases, but from a new point of view, inside the function of thyroid hormones and with a systematic vision, involving diseases affecting other organs than thyroid.

Three different sessions chaired by Prof. Domenico Salvatore and Prof. Giovanni Levi, have deepen the specific roles of thyroid hormone machinery in different tissues, as liver, brain, sensory organs, bone, skeletal muscle and even in the reproductive response to seasons.

The subjects were developed by Prof. Theo Visser (Internal Medicine, Erasmus University Medical Center, Rotterdam), Dr. Elizabeth McAninch (Division of Endocrinology and Metabolism, Rush University Medical Center, Chicago), Prof. Takashi Yoshimura (Institute of Transformative Bio-Molecules, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya; Division of Seasonal Biology, National Institute for Basic Biology, Okazaki), Prof. Douglas Forrest (Laboratory of Endocrinology and Receptor Biology, NIDDK-NIH, Bethesda), Prof. Barbara Demeneix (Muséum National d'Histoire Naturelle, CNRS, Paris), Dr. Jens Mittag (Center of Brain, Behavior and Metabolism, Lübeck), Prof. Warner S. Simonides (VU University Medical Center, Amsterdam), Dr. Monica Dentice (Department of Clinical Medicine and Surgery, Federico II University, Naples) and Prof. Graham R. Williams (Molecular Endocrinology Laboratory, Department of Medicine, Imperial College London, London).

The Forum focused on the new perspective of considering the local action of thyroid hormones as a possible therapeutic approach for various diseases.

# Introduction

**Domenico Salvatore**

Department of Molecular and Clinical Endocrinology, Federico II University, Naples, Italy

Thyroid hormone exerts a wide spectrum of biological effects in vertebrates via nuclear thyroid hormone receptors. These effects are mainly mediated by the active form of thyroid hormone, namely, T3. Thyroid hormone signaling is a consequence of the interaction of T3 with nuclear receptors that, together with other transcription factors, stimulate or repress the expression of target genes.

Local thyroid hormone action is regulated by a complex cascade of processes, i.e., T4 and T3 uptake (via thyroid hormone transporters), local thyroid hormone activation and degradation (via deiodinases), T3 transport/diffusion to the nucleus, binding to nuclear thyroid hormone receptors (TRs), and interaction of theT3-TR complex with co-repressors and co-activators.

A large body of evidence indicates that the control of TH action at peripheral level significantly affects the physiology of many organs and tissues. It has long been recognized that circulating thyroid hormone levels do not faithfully reflect TH status in several cell types. Tissue-specific regulation of the components of this system enables precise temporal and cell-specific control of thyroid hormone action irrespective of circulating thyroid hormone levels.

Although knowledge is still primitive, recent data raise the possibility of therapeutically targeting local thyroid hormone metabolism to modify TH action in selected tissues. A crucial issue regarding the therapeutic potential of this potent hormone is how to differentially modulate its action in a time- and tissue-specific manner in order to avoid the deleterious effects of excessive T3. In this context, manipulation TH metabolism is a promising theoretical approach since it can increase or decrease thyroid hormone signaling irrespective of thyroid hormone serum concentrations. Thus, the spatio- and temporally-controlled regulation of TH action would allow for the modulation of the complex pattern of specific TH-sensitive gene expression in diverse

developmental and disease states. Obviously, elucidation of thyroid hormone action at cellular level is a prerequisite for the use of deiodinases or other means for therapeutic purposes. Mouse models of tissue-specific knock-outs and over-expression of deiodinases will reveal how these enzymes impact TH signaling in selected tissues and organs, and pave the way for their therapeutic exploitation.

In this scenario, the central idea of this meeting was to discuss the thyroid gland, not looking at thyroid-related diseases, as is generally the case, but from a new stance, namely, systematically within the function of thyroid hormones, unraveling the mechanisms by which thyroid hormone affect organs other than the thyroid, and how these effects could be manipulated in a therapeutical context.

The aim of the Forum is to gather together leading experts in the field of thyroid hormone action to stimulate discussion, collaborations, new research and last but not least friendship among participants.

# SESSION 1

## THE THYROID… IN THE PERIPHERY

# Pathophysiological importance of thyroid hormone transporters

**Theo Visser**

Internal Medicine, Erasmus University Medical Center, Rotterdam, The Netherlands

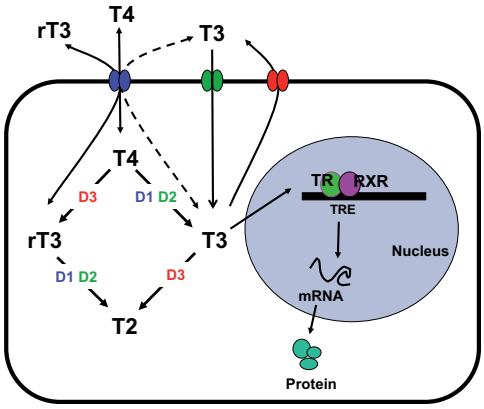
Thyroid hormone (TH) exerts its biological activity largely through binding of the active hormone T3 to nuclear receptors, leading to changes in the transcription of target genes [1]. Furthermore, the deiodination processes involved in the production or degradation of T3 also occur intracellularly [2]. Therefore, cellular uptake of TH is crucial for its action and metabolism. TH transport in and out of cells is facilitated by transporters, belonging to two different categories: the organic anion transporters (NTCP, Na/taurocholate cotransporting polypeptide and OATPs, Organic anion transporting polypeptides) and the amino acid/monocarboxylate transporters (LAT1,2, L-type amino acid transporters, and MCT8,10, monocarboxylate transporters) [3].

MCT transporters mediate the transport of several diverse molecules. MCT8 specifically transports both thyroid hormones T3 and T4, while MCT10 transports primarily T3 and in addition phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) [4,5].

MCT8 and MCT10 are homologous transporters encoded by homologous genes. Their amino acid homology is particularly high in the 12 transmembrane domains (• **Figure 1**).

The expression of MCT8 and MCT10 in human tissues is different: MCT8 is well represented in brain, kidney, liver and thyroid gland, while MCT10 is highly expressed in skeletal muscle [6]. In the brain MCT8 is expressed importantly at the apical and the basal lateral membrane of the endothelial cells of the blood brain barrier [7-9], where it facilitates the transcellular transport of T4 and T3. In humans, OATP1C1 facilitates the uptake of T4 in astrocytes [10]. Once in the cell the hormone is converted to active T3 by deiodinase D2; T3 is then released in the extracellular space, becoming available for uptake into neurons and oligodendrocytes. MCT8 is also involved in neuronal T3 uptake [11]. It is then evident that MCT8 plays a crucial role in brain development.

• **Figure 1.** Thyroid hormone transport and metabolism in T3 target cell




Allan-Herndon-Dudley syndrome (AHDS) patients are characterized by central hypotonia, poor head control and distal dystonia, severe retardation, reduced body length and weight and delayed myelination [12-14]. Male AHDS patients have been analyzed for MCT8 mutations in different laboratories and over hundred mutations, including deletions, nonsense mutations, frameshift mutations, and missense mutations have been discovered so far.

Thyroid function tests (TFTs) in AHDS patients are abnormal; mean TSH is modestly increased, FT4, T4 and rT3 are markedly decreased, and T3 is markedly increased. The T3/rT3 ratio is thus highly elevated in AHDS patients. TFTs tend to be less abnormal in patients with a milder phenotype who are capable of speaking and/or walking although with great difficulty.

We analyzed the effect of missense mutations in MCT8 by looking at T3 transport in transfected JEG3 cells. Most tested mutations resulted in an almost complete inactivation of MCT8. However, a number of mutations were associated with significant residual T3 transport. This was especially the case for mutations identified in patients with a milder phenotype. However, T3 transport activity overlapped with residual activity of MCT8 mutants from severely affected patients, suggesting that a genotype-phenotype correlation is not clear.

In conclusion, it was found that there is a modest correlation between severity of illness, serum TH levels and residual MCT8 activity in AHDS patients. Therefore it may be hypothesized that other important molecules than TH are transported by MCT8. We have therefore tested if MCT8 is capable of transporting amino acids as has been demonstrated for MCT10. However, extensive studies have excluded the possibility that MCT8 facilitates transport of amino acids, including the aromatic amino acids Trp, Phe and Tyr, which are substrates for MCT10. Further studies are ongoing in our laboratory to identify alternative substrates for MCT8, if they exist.

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# Local thyroid hormone activation in the liver defines susceptibility to obesity

**Elizabeth McAninch**

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The thyroid gland produces thyroid hormones in both the pro-hormone form, T4, and the active form, T3. The hormones are then released in the blood. T3 levels in circulation are known to be extremely stable during adult life, except in the case of severe illness or fasting, although the metabolic requirement of the cells is very variable. This suggests that peripheral systemic levels of T3 are not directly responsible for those rapidly fluctuating for metabolic changes within the cells. The key players in regulating the inner levels of iodothyronine are the deiodinases, D1, D2 and D3, located within the cell.

One important target of T3 is the liver, a classic example of a T3-dependent tissue. In the liver T3 stimulates lipogenesis, fatty acid oxidation, bile acid synthesis, and cholesterol metabolism. In the case of thyrotoxicosis hepatic glucose uptake is increased, while hepatic insulin sensitivity is decreased. It is also known that liver is an important site of TH clearance.

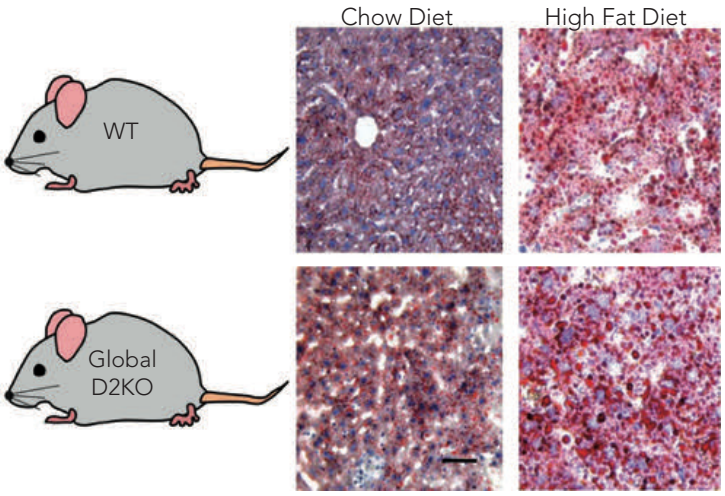
Considering the relevance of T3 action in the liver, many scientists have wondered about the importance of the type 2 deiodinase in this organ.

As far as it is known, this enzyme is expressed in the liver in chickens, in amphibians, in the macrophages within the liver of mice and ectopically in the liver X receptor knockout mice, but there is no D2 in the adult mammalian liver.

The objectives of the study were to characterize the phenotype of a mouse with the specific inactivation of the type 2 deiodinase within its hepatocytes, and to define the metabolic impact of D2-mediated T3 production in the liver throughout mouse development and adulthood.

We first compared D2 global knock out mice to wild type mice on a chow or a high fat diet [1]. Interestingly there was a marked fat accumulation in the D2 knockout mouse liver, which was more affected by steatosis, as compared to the wild type mice (• **Figure 1**).

• **Figure 1.** Global D2KO




*Source:* Castillo *et al.*, 2011 [1].

Then a mouse with a specific hepatocyte D2 knock out was created: the ALB-D2KO mouse. No differences within T3 and T4 serum levels, body weight evolution, body composition and liver histology were observed between the ALB-D2KO mice and the control mice. However, when the mice were put on a high fat diet the ALB-D2KO mice did not gain extra weight and they were protected from diet-induced obesity. In particular, the body composition was very different. On a high fat diet, the ALB-D2KO mice showed reduced fat percentage, reduced epididymal weight and reduced liver weight. Even the cholesterol levels were less increased, while triglycerides levels did not increase at all, showing that ALB-D2KO mice are protected from hypercholesterolemia, too.

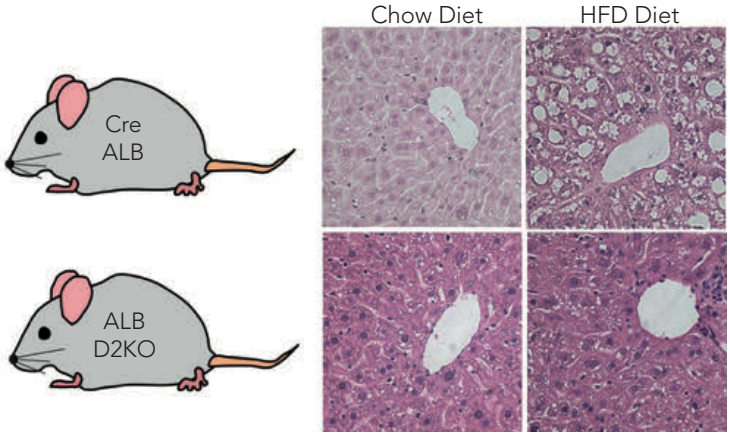
When livers were analyzed, it was evident that fat levels in WT and ALB-D2KO mice were comparable on a chow diet, while on a high fat diet no increase in the fat in the liver was observed in the ALB-D2KO mice. Moreover, liver cholesterol and triglycerides in the ALB-D2KO mice did not show such a significant increase as the wild type mice [2] (• **Figure 2**).

In summary, liver specific D2 inactivation prevents diet-induced liver steatosis, obesity and hypertriglyceridemia.

However, considering that D2 is not expressed in the adult mouse liver, it was conceivable that the loss of D2 dependent processes during liver development was responsible for the observed phenotype.

The study of D2 mRNA in fetal and neonatal liver revealed that mRNA expression reaches a maximum at post-natal day 1 (P1) and then falls in a few days to become undetectable.

• **Figure 2.** ALB-D2KO Phenotype




*Source:* Fonseca *et al.*, 2015 [2].

D1 and D3 mRNA are expressed in P1 mice liver as well, then while the first one increases until adult life, D3 shows a trend similar to D2, becoming barely detectable in the adult.

The D2 activity in the neonatal mice livers was completely abolished in the global D2KO mice, while, impressively, there was some residual activity in the ALB-D2KO mice. Interestingly, the D1 and D3 mRNA expression levels in the ALB-D2KO mice were comparable to those in the control mice, unlike D2 mRNA which showed a peak which was reduced by half in P1 mice [2]. This decrease in D2 levels translates in a decrease in T3 production and liver content (• **Figure 3**).

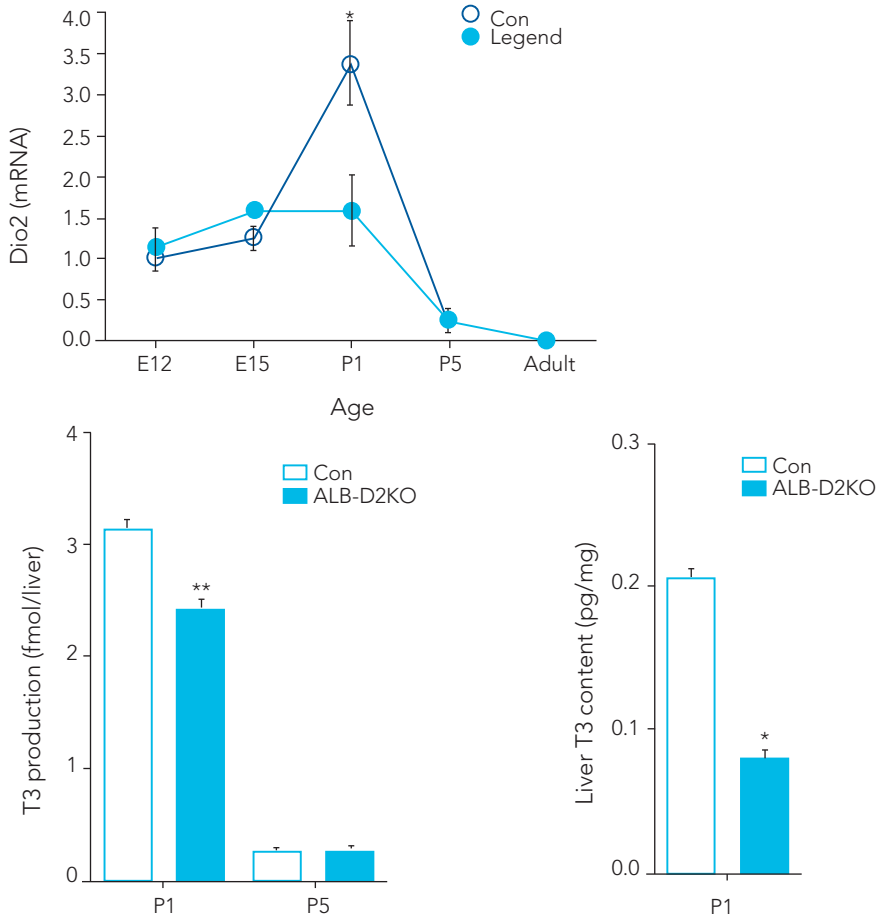
To analyze the mechanisms underlying the cited effects, some candidate lipid homeostasis genes have been analyzed and found to be significantly reduced in the ALB-D2KO mice at P1. Interestingly the discrepancy completely disappeared in the adult, suggesting that the different adult phenotype we observed at the beginning could be due to the P1 gene expression pattern.

A transcriptome analysis in the liver of ALB-D2KO and control adult mice on a chow diet showed 165 genes with significantly differing expression levels. These genes were related to: lipoprotein binding, carbohydrate transport, inflammation, cell signalling and structure, DNA and cell cycle, methyltransferase and sulfurtransferase.

We then hypothesized that the presence or the absence of perinatal D2-generated T3 in hepatocytes permanently modifies gene expression and defines a phenotype that is carried forward through adulthood.

Moreover, when the DNA methylation profile was analyzed, about 3 thousands differentially methylated local regions (DMRs) were identified in ALB-D2KO mice.

• **Figure 3.** T3 is reduced in ALB-D2KO liver

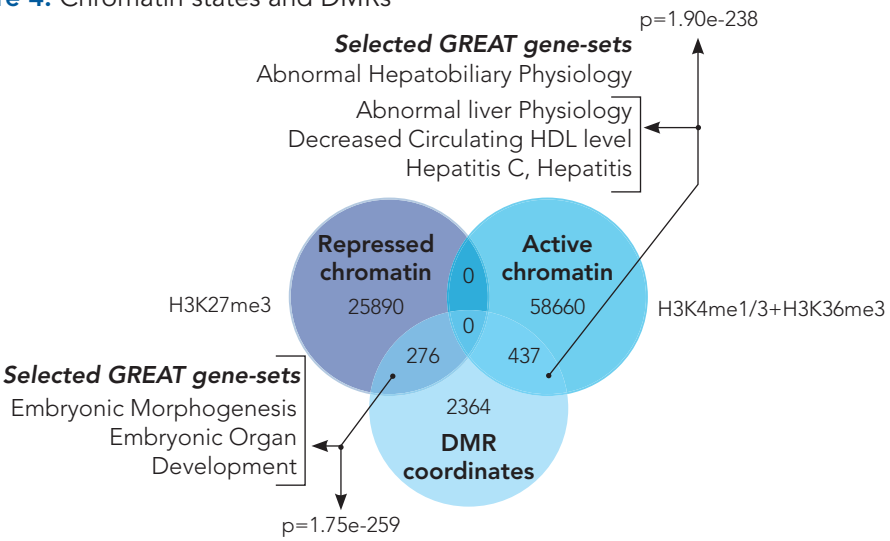



*Source:* Fonseca *et al.*, 2015 [2].

DMRs located in the active chromatin belong to liver-related gene sets as hepatobiliary physiology, liver physiology and hepatitis. Regions located in the repressed chromatin were related to embryogenesis and organ development, instead (• **Figure 4**).

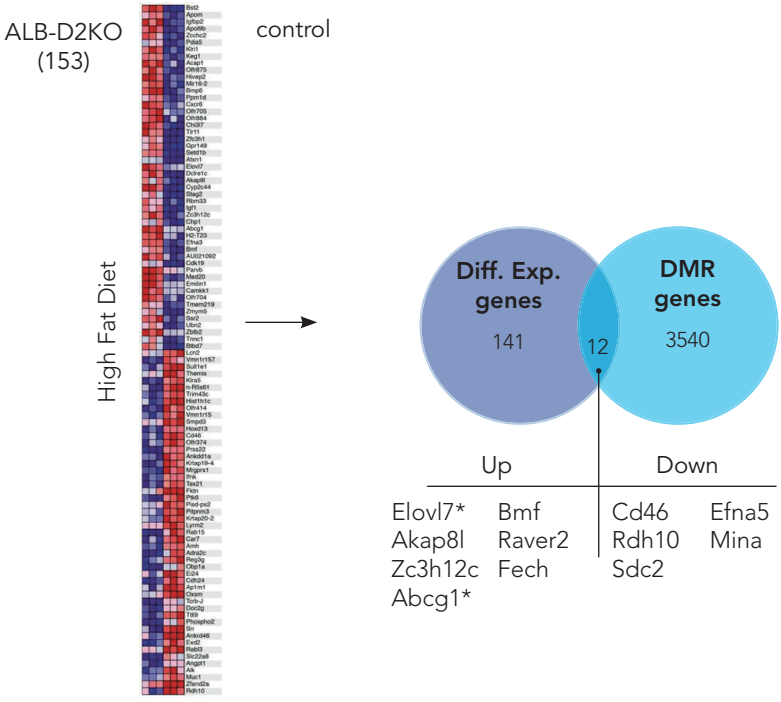
We then moved to the analysis of ALB-D2KO and control mice on a high fat diet, and found that the induction of genes controlling lipid metabolism was impaired. When transcriptome and DNA methylation analyses were conducted, the affected genes and DMRs were completely different from the ones found on a chow diet, meaning that the diet influences the gene expression. On a high fat diet about 10% of regulated genes contained affected DMRs (• **Figure 5**).

• **Figure 4.** Chromatin states and DMRs




*Source:* Fonseca *et al.*, 2015 [2].

• **Figure 5.** Transcriptional differences on HFD; DNA methylation




*Source:* Fonseca *et al.*, 2015 [2].

In conclusion D2 is briefly expressed in neonatal mouse hepatocytes. However the disruption of this D2-mediated T3 signaling protects adult mice from diet-induced obesity, steatosis and hypertriglyceridemia; reduces the expression of genes in neonatal liver involved in fatty acid, triglyceride, and cholesterol synthesis, and permanently modifies the liver transcriptome via epigenetic mechanisms that involve DNA methylation.

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# Thyroid hormone and seasonal regulation of reproduction

**Takashi Yoshimura**

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Various physiology and behaviors are seasonally regulated in animals, among them reproduction is one of the most important for human culture. In calendar of seasonal breeding, most short and long day breeder animals are genetically programmed to anticipate the seasons and mate in time to give birth in spring, when food is becoming plentiful and temperatures are becoming warmer [1]. Animals are then able, somehow, to sense seasons.

In vertebrates, reproduction is regulated by the hypothalamus-pituitary-gonadal (HPG) axis, hence GnRH secretion is seasonally regulated. In mammals light information is received by the eye, and transmitted to the suprachiasmatic nucleus. The cycle of light and dark reflects on melatonin production, which regulates GnRH secretion in a way which has not been clarified yet. Interestingly in birds, despite the presence of melatonin, this hormone has no impact on GnRH production; nonetheless birds do not require eye for seasonal sensing, which is received directly by brain.

To clarify the mechanisms underlying these observation, we chose Japanese quail as a model. This bird presents evident phenotype changes in response to seasons, testis size, for example, is influenced by long or short day periods. In birds the mediobasal hypothalamus (MBH) is a center for photoperiodic time measurement then we focused on MBH. It was previously demonstrated [2] that light pulses given at photo-inducible phase cause testicular growth.

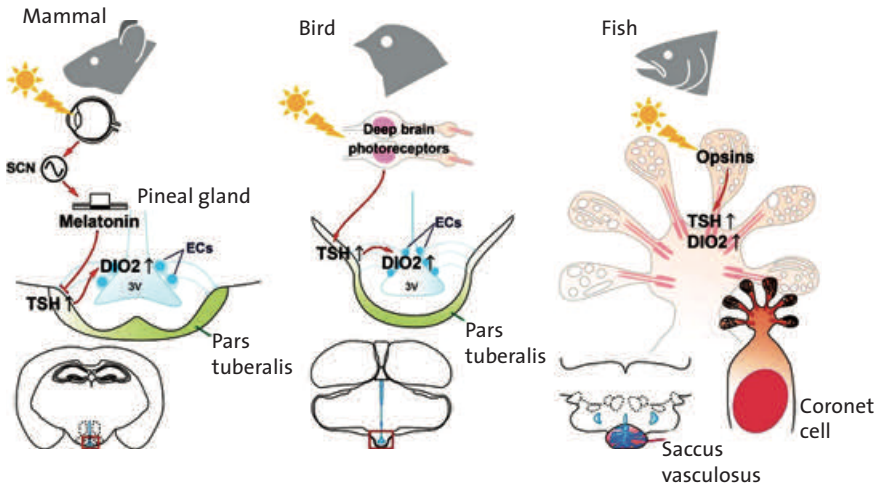
We chose this experimental model to do some differential analyses, and found the long day induction of type 2 deiodinase (DIO2) gene within the MBH [3], causing an increase in T3 concentration in that zone. Since thyroid hormone is essential for central nervous system development and plasticity, we studied GnRH nerve terminal and the end feet of glial processes and observe morphological changes T3 responsive

which can be involved in the responsiveness to seasons [4]. When bird genome became available, we could perform functional genomic analysis of quail photoperiodism and found that DIO2 and DIO3 (type 3 deiodinase) are oppositely regulated. Long day stimulus induces TSH in the pars tuberalis, which activates DIO2 in turn [5] through the TSHR-Gsα-cAMP signaling pathway.

We also demonstrated TSH-TSHR involvement in mammals, when melatonin was administered to TSHR knock out mice no effect was observed either on DIO2 (decreased in wt mice) or DIO3 (increased in wt animals) expression. This suggest that TSH-TSHR is involved in mammalian seasonal reproduction [6].

Differences between birds and mammalian melatonin response are critical. While in mammals the hormone is essential, birds do not require melatonin for seasonal reproduction, and their response to light do not involve eyes. We then decided to identify brain photoreceptors. Since many different opsin receptors are present in vertebrates, even if we still do not know the function of every one, we decide to investigate whether some of them could be represented in quail. Indeed, we found that OPN5 is expressed in the cerebrospinal fluid (CSF)-contacting neurons within the paraventricular organ (PVO), it was expressed in the bipolar neuron. Moreover we also confirmed that opsin 5 is involved in light sensitivity [7], and it can be considered a brain photoreceptor. This consideration is quite obvious from a deve-

• **Figure 1.** Signal transduction pathway regulating seasonal reproduction in vertebrates




*Source:* modified from Nakane, Yoshimura, 2014 [9].

lopmental point of view, considering that retina and pineal organ evaginate both from the subventricle where OPN5 positive CSF contacting neurons are locate [8]; we then can speculate that these neurons are ancestral photoreceptors in vertebrate. We can then uncover the signaling transduction pathways involved in seasonal reproduction in birds, confirming the involvement of TSH in mammals and in birds.

Interestingly, thyroid hormone are important even in fish, despite the fact that fish do not have pars tuberalis. Thus, we decide to identify the tissue which is responsible for seasonal reproduction in fish. As a first step we analyzed different tissues for the expression of beta-subunit of TSH and DIO2, and found seasonal changes in these genes in the saccus vasculosus, a fish specific organ which function is still unclear. When we analyzed the function of this organ, we found it to be a seasonal sensor in fish [9] (• **Figure 1**).

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## SESSION 2

# THE THYROID… IN THE BRAIN

# Thyroid hormone and sensory development

**Douglas Forrest**

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Thyroid hormone (T3) is known to regulate sensory development and sensory defects, particularly in auditory function are related to diseases, such as resistance to thyroid hormone or developmental disorders such as hypothyroidism and iodine deficiency [1]. However, very little is known about the influence of thyroid hormone on the visual system.

In the retina, different types of photoreceptors mediate responses to different wavelengths of light. In particular, cone photoreceptors are responsible for vision in bright light and colour vision, while rod photoreceptors are responsible for dim light vision.

Most mammalian species are dichromatic and have two different types of cone photoreceptors, expressing M and S opsins, for response to medium-long ("green") and short ("blue") wavelengths of light, respectively. M and S cones are distributed in gradients over the retina: M in the superior and S in the inferior.

We have determined that concerning thyroid hormone receptor expression, TRβ2, a receptor isoform encoded by the *THRB* gene, is uniquely expressed in the cone photoreceptors. TRβ2 has been shown to be critical for M cone development. In TRβ2 knock out mice, green light response is lost, due to lack of M cones. We found that TRβ2 is expressed in photoreceptor precursors, and is essential for the diversification of cones into S and the M phenotypes [2].

These findings suggest an unexpectedly critical role for thyroid hormone in cone photoreceptor function.

Deiodinases are well known selenoenzymes implicated in thyroid hormone homeostasis [3, 4]. In particular, the type 3 enzyme, Dio3, inactivates T3, and in the absence of Dio3, tissues are exposed to T3 excess. We found that Dio3 is highly expressed in the retina at the early stages of development during embryogenesis

then declines later during postnatal development, when the retina differentiates. It is therefore conceivable that Dio3 expression plays a role in retina development. Indeed in Dio3 knock out mice, we observed a severe loss of both M and S cone photoreceptors, with a consequent achromatopsia, that is, a loss of visual function in bright light conditions [5].

Cones are generated in Dio3 knock out mice, but an apoptotic process is then triggered by exposure to excess of T3, causing cone loss. This is also evident in wild type mice exposed to supraphysiological doses of T3, which eliminates cones by apoptotic death. In addition, we demonstrated that the TRβ2 receptor isoform is essential for this loss of cones; in fact, when TRβ2 is deleted in the Dio3 knock out mice, no loss of cones is observed. It is then conceivable that TRβ2 can promote cone diversity and survival in the presence of the right amount of T3, while it causes cone apoptosis in an excess of T3. Hence deiodinase 3 acts as a sensor, able to constrain the T3 signal to safeguard cone differentiation and long-term survival.

These observations may have implications in human disease although only a few human cases have been studied so far. In rare cases of thyroid hormone resistance syndrome, monochromacy and loss of long wave sensitivity has been observed, suggesting a possible conserved function for the *THRB* gene in cone function in the human retina. No data are available concerning mutations in the human DIO3 gene, so far. A future question is whether it is possible to use these findings to counter photoreceptor loss in retinal degeneration [6].

Retinal diseases such as age-related macular degeneration, cone dystrophies and retinitis pigmentosa, can affect cone photoreceptors. These diseases are characterized by genetic heterogeneity [6]; multiple genes can cause photoreceptor death by triggering pre-apoptotic signals that converge on apoptotic pathways. Potentially, reducing T3 exposure might reduce the signals that lead to the death of cone photoreceptors. Interestingly, when hypothyroidism is induced in some mouse models of retinal degeneration, a partial preservation of cone photoreceptors has been reported [7].

We propose that TRβ2 is central to the life history of cone photoreceptors and that it influences cone diversity, maturation, survival and degeneration. Detailed investigation of cone photoreceptors has been limited because of the small numbers of cones present in the retina in mouse models. Cones represent only 3% of photoreceptors and are out-numbered by rods which represent 97% of retinal photoreceptors. In ongoing studies, we have designed techniques to isolate cones for transcriptome analysis and to identify candidate target genes that are regulated by TRβ2 during cone differentiation.

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# Thyroid hormone and neural stem cells: from brain evolution to endocrine disruptors

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Thyroid hormones (THs) levels are crucial for normal development of the central nervous system. Without the right amount of thyroid hormones at the right time, humans suffer from cretinism. Since thyroid hormones play essential roles in early neurogenesis, the discovery of neural stem cells in adult mammalian brains led to the valid question of whether thyroid hormones and thyroid hormone receptors (TRs) were implicated in adult neural stem cell control.

Neural stem cells are located in two main sites of the adult brain: the subgranular zone of the hippocampus (SGZ), where neurogenesis is implicated in generated new neurons necessary for memory, and the subventricular zone (SVZ) of the lateral ventricle, where the new neurons generated migrate to the olfactory bulb and are implicated in smell. It is interesting that in hypothyroidism both these functions, olfaction and memory, are affected, an observation that could already suggest that thyroid hormones and their receptors might be implicated in processes that depend on adult neurogenesis.

Our work has focused on the neural stem cell niche in the subventricular zone, where neural stem cell (NSC) give rise to transit amplifying progenitors (TAPs), TAPs can evolve either in a neuronal precursor cell (NPC) a first step in the neuroblast lineage, or into a oligodendrocyte precursor cell (OPC), engaging in the oligodendrocyte lineage. NPCs can then differentiate into mature neurons and OPCs into mature oligodendrocytes.

A first question was which thyroid hormone receptors were expressed in the NSCs and TAPs. Immunofluorescence experiments revealed that only TRα is expressed in the subventricular zone, while TRβ is expressed outside the SVZ. About four years ago we reported that TRα1 expression appears in TAPs and, as it increases, it downregulates Sox2, a key gene in pluripotency. In particular, we observed that the thyroid

hormone signaling actively represses Sox2, via TRα1 [1]. In addition, T3/TRα1 also downregulates cyclin D1, cMyc and surprisingly, EGFR, a gliogenic factor in the glial lineage, which is expressed in oligodendrocytes. It is conceivable that some additional players might be involved to protect oligodendrocytes from T3 induced EGFR repression.

To clarify how EGFR positive cells are "protected" from the neurogenic effects of T3, we carried out loss of function or a gain of function of TRα1 directly in the brain. These experiments confirmed that T3/TRα1 signaling downregulates the gene encoding EGFR. We then focused on the deiodinase 3 (D3) expression, and observed that this was highly expressed not only in stem cells but also in the proliferating EGFR+ oligodendrocyte progenitors, but not in the DCX+ neuroblasts.

We wondered whether these findings might have applications in therapeutic settings, we thus decided to test them in a model of demyelination, specifically an animal model of multiple sclerosis. To this end, mice were demyelinated in the corpus callosum and simultaneously exposed to treatments to induce hypothyroidism. Looking at the distribution of myelin basic protein, we observed an increase of its expression in the hypothyroid demyelinated mice versus euthyroid demyelinated ones. Furthermore, myelinated axons in hypothyroid demyelinated mice were comparable to the control ones, meaning that myelination was restored by transient hypothyroidism.

These findings led us to ask which other mechanisms, besides inactivating deiodinases and receptors, could determine differences in thyroid hormone availability between OPCs and NPS and thus affect cell fate decision. Obvious candidates are thyroid hormone transporters, as well as deiodinase 2 (an activating deiodinase); very little is yet known about distribution of either of them in neurogenic areas.

Moreover, cell metabolism might be involved. It is worth bearing in mind that stem cells and differentiated cells have different metabolic capacities (aerobic glycolysis versus oxidative phosphorylation) and that stem cells have virtually no mitochondrial activity. Since it is well known that thyroid hormones modulate metabolic responses both at the level of the whole organism and at the cellular and mitochondrial levels, we can reasonably ask what sequence of cellular events occur as a function of increased thyroid hormones entering the cells. Given the differential distribution of TRα in NPCs and OPCS we are currently addressing these questions and more specifically there is a thyroid hormone dependent difference in mitochondrial activity within these cell types.

It is known that brain evolution is characterized by myelin acquisition and changes in glia/neuron ratio, both these characteristics being affected by thyroid hormones. What is more, recent data show that small variations in maternal thyroid hormone levels affect children's IQ and brain structure [2]. Brain development consists of a suite of different events, including progenitor amplification, migration, lineage decision, differentiation, myelination, synaptogenesis. All of these processes are modulated by changes in thyroid hormone availability. It is then reasonable to ask if the

long-term environmental exposure to multiple thyroid hormone disruptors, many of which have been found in the amniotic fluid, is affecting brain development [3]. Strengthening this concern is the observation that incidence of neurodevelopmental disease (both Autism Spectrum Disorders and Attention Deficit/Hyperactivity Disorders) have increased of 25 fold in the last 15 years [4].

Another argument potentially linking these increases to thyroid hormone disruption is that maternal hypothyroidism increases the risk of neurodevelopmental disease.

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# Role of thyroid hormone in brain development and function

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Thyroid hormones are really important for brain development, since when thyroid hormone signaling is impaired during brain development, mental retardation occurs. However, little is known about the genetic and neuroanatomical targets of thyroid hormones in the brain.

The main question is then how thyroid hormones act in the brain.

It has been demonstrated that the thyroid hormone receptor alpha 1 (TRα1) is the main isoform in the brain, being expressed in the majority of postmitotic neurons [1]. Its distribution suggests a role in the processes of migration and differentiation of neurons.

To highlight the specific role of TRα1 in the brain, a mutant mouse with a mutation in the T3 binding site of TRα1 was studied [2], these TRα1+ mice represent a model for TRα1 mediated hypothyroidism. The mutant receptor can be reactivated by T3 treatment during any period in life, allowing differentiating between developmental and acute actions of TRα1. In particular, the anxiety of these animals can be rescued by T3 treatment in the adult mice, while motoric function, depending on brain development, can be reverted only when T3 treatment takes place between postnatal day 10 and 35.

Parvalbumin neurons have been identified in the recent years as primary targets of TRα1 action.

They are located in the cortex, an area involved in sensation and voluntary movements, in the hippocampus, where they are connected to memory and spatial navigation, and in the hypothalamus, where their role has not yet been clarified.

Interestingly, precisely in this last location, we have observed a reduction of parvalbumin neurons in the TRα1 mutant mice, which cannot be reverted by adult T3 treatment, suggesting a role at the developmental stage for TRα1 [3]. When TRβ1 is

inactivated too, the number of parvalbumin neurons goes down further, suggesting that both the receptor isoforms are involved in the development of these neurons. To investigate the role of these neurons we generated a mouse model with a strong reduction of parvalbumin cells in the AHA (anterior hypothalamic area); these animals did not show any significant change in metabolism, but they showed a different cardiovascular phenotype. The animals with AHA pv+ cell ablations showed prominent hypertension, with increase in both systolic and diastolic blood pressure; in addition the heart rate was also elevated with bigger differences at lower temperatures [3].

Recently, it was demonstrated that these findings could be of some relevance for human beings. A very elegant study [4] correlated maternal hypo and hyperthyroidism with elevated blood pressure in the offspring, confirming the connection between thyroid hormone levels during development and hypertension later in life.

So far molecular mechanisms of the action of thyroid hormone on parvalbumin neurons are unknown. It seems likely that thyroid hormone acts in different developmental windows on cortical and AHA parvalbumin neurons, since cortical and AHA parvalbumin neurons are not coming from the same origin. Unfortunately, we the target genes of thyroid hormones in these processes have not been identified to date.

Considering the heterogeneity of the brain, answering this matter could represent a tough question. The TRα1-GFP mouse [5] could be helpful in this context to isolate TRα1 bound chromatin using a GFP antibody [6], and compare samples from euthyroid and hypo and hyperthyroid animals to specifically identify target genes of TRα1.

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## SESSION 3

# THE THYROID... IN BONE AND MUSCLES

# The role of cardiac thyroid hormone signaling in heart failure

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Thyroid hormones enter the cell through specific transporters and, once in the nucleus, their binding to a thyroid hormone receptor regulates specific gene expression, such as SERCA2, RYR, MHCα and -β in cardiomyocytes. The heart is one of the main targets of thyroid hormone and cardiovascular function is greatly affected in thyroid disease. In particular heart rate, ejection fraction and rates of contraction and relaxation are all sensitive to thyroid hormone levels.

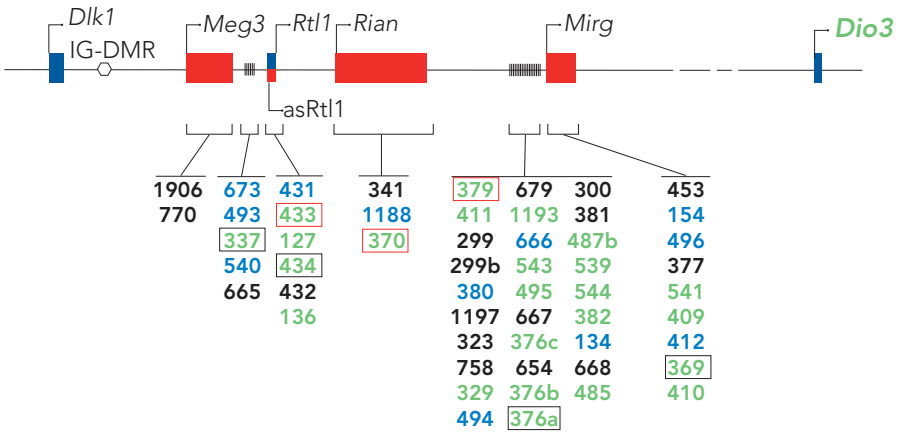
Heart failure is a progressive disorder of ventricular remodeling induced by chronic hemodynamic overload, culminating in a clinical syndrome characterized by impaired cardiac function and circulatory congestion. It can be caused by pressure overload – as a consequence of pulmonary hypertension or aorta stenosis –, by ischemic heart disease and by loss of viable myocardium due to infarction. All these conditions are able to induce pathological ventricular hypertrophy and remodeling, that can eventually fail in maintaining a proper cardiac function. In heart failure specific genes are affected and many of those are similarly altered in hypothyroidism [1].

We investigated a mouse model of myocardial infarction (MI) and found that tissue content of T3 was decreased by 50%, while deiodinase type 3 activity (Dio3) was substantially increased, with most of the cardiomyocytes expressing this enzyme [2]. Many studies, using different models of heart failure, have confirmed that Dio3 is induced in stressed myocardium, leading to a local hypothyroid condition and affecting T3-regulated gene expression.

Although various factors may potentially regulate Dio3 transcription, the mechanism of up-regulation of Dio3 expression in the stressed heart is unknown. We analyzed microRNA (miR) expression in the post-MI mouse heart as a possible factor in this mechanism. Surprisingly, a large group of miRs located in the Dlk1-Dio3 genomic region was up-regulated in cardiomyocytes (coloured numbers in • **Figure 1**), together

with Dio3 mRNA [3]. Up-regulation of these miRs in stem cells is a signature of pluripotency and proliferation. Similarly, various tumors express these miRs in association with Dio3. It appears that a proliferative program is initiated in the heart in response to MI and that up-regulation of Dio3 expression and reduction of T3 is an aspect of this program. However, although neonatal cardiomyocytes are able to de-differentiate and proliferate, this capacity is lost in adult cardiomyocytes. It may have therapeutic implications to establish what limits the regenerative program in the adult heart.

• **Figure 1.** Post-MI up-regulation of microRNAs in the Dlk1-Dio3 genomic region

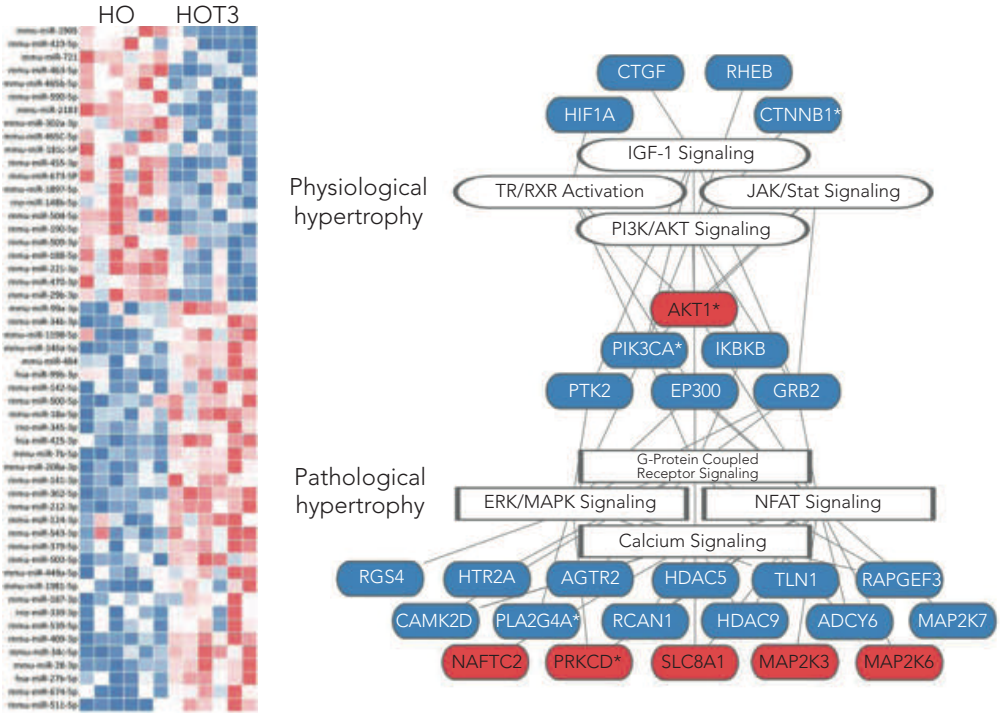



*Source:* Janssen *et al.*, 2013 [3].

Both pathological and physiological hypertrophy are regulated by a complex net of signal-transduction pathways. The physiologic ones are known to be targeted by thyroid hormone, yet nothing is known about the possible involvement of T3-regulated miRs. To investigate this, we analyzed the effect on cardiac miR expression of a short T3 exposure of hypothyroid mice and found that 52 miRs were significantly up- or down-regulated by more than a factor of 2 [4]. Surprisingly, the majority of the predicted effects of these changes was inhibition of pathological hypertrophy, and only some stimulation of physiological hypertrophy (• **Figure 2**).

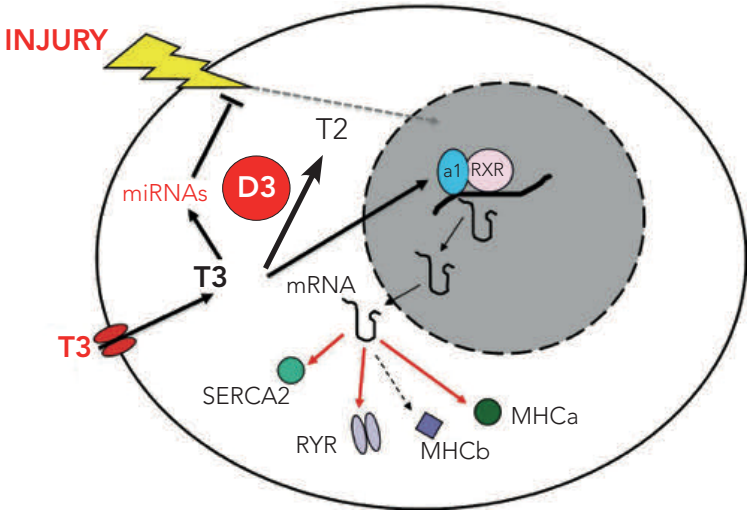
It would appear that T3 puts the brakes on pathological hypertrophic pathways, which could explain why a chronic increase in hemodynamic load induced by T3 leads to physiological, instead of pathological hypertrophy. Conversely, this brake will be released as a result of the local hypothyroid condition in heart failure, aggravating the pathological remodeling (• **Figure 3**).

• **Figure 2.** T3-regulated microRNAs




*Source:* Janssen *et al.*, 2014 [**4**].

• **Figure 3.** Consequences of Dio3 activity in heart failure



Unpublished results of analyses of 75 donor hearts and 36 explanted failing hearts indicate that Dio3 is also induced in human ischemic heart disease and associated with a reduction of T3 levels in the failing left ventricle.

We can conclude that T3 is able to put a brake on pathological hypertrophic signaling through the action of miRs. Reduction of cardiac T3 levels as a result of increased Dio3 activity will therefore promote pathological remodeling through both direct transcriptional effects and indirect translational effects mediated by miRs.

Recent animal and human studies have yielded conflicting results concerning the efficacy of T3 treatment in heart failure. Targeting Dio3 may be a better therapeutic option if this enzyme is indeed highly active in the stressed heart.

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# Thyroid hormone metabolism in muscle physiology and disease

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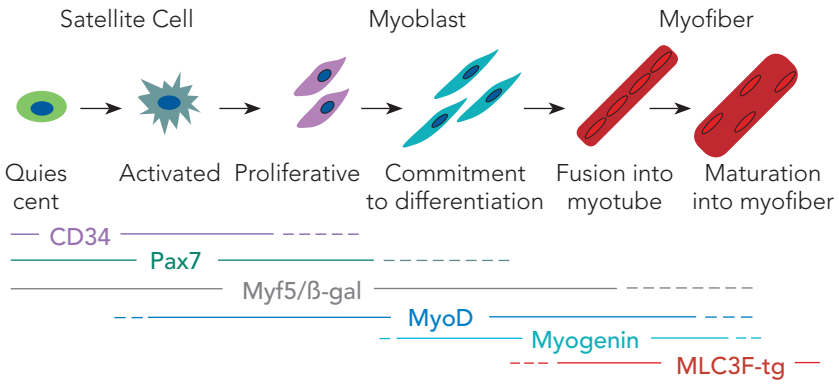
Skeletal muscle is a well known target for thyroid hormone action. Thyroid hormones, in fact, are able to affect composition, contraction, strenght, energy homeostasis and glucose metabolism in skeletal muscle; moreover specific skeletal muscle genes are also regulated by thyroid hormones. It is not surprisingly, then, that myopathies often occur in thyroid dysfunction.

A novel target for thyroid hormone action are satellite cells. These skeletal muscle stem cells are regulated in quiescence, proliferation and differentiation by thyroid hormones. Satellite cells are localized at the basal lamina of myofibers, in a quiescente state, and are activated in case of damage and recruited by myofibers. They also are

• **Figure 1.** Lineage progression of satellite cells

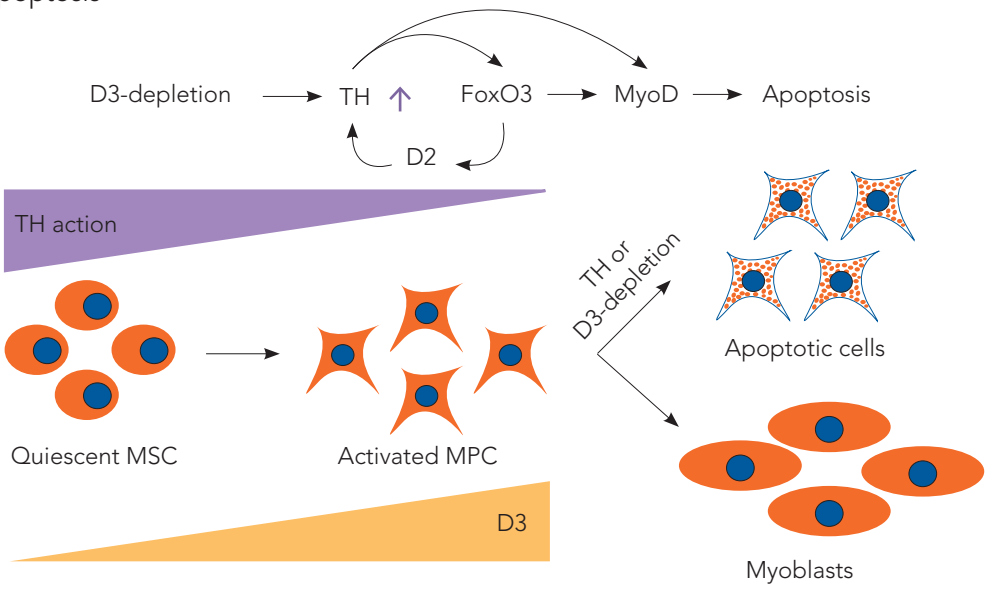



*Source:* Bentzinger *et al.*, 2014 [1].

able to return in a quiescent state to replenish the satellite cell niche. The lineage progression of satellite cells (• **Figure 1**) can be regulated by thyroid hormones, which can intervene by regulating the expression of some major genes involved in the control of myogenesis [1].

In the recent years our group has been focused on the local modulation of thyroid hormones in skeletal muscle, by studying the selenoenzymes deiodinases D2 and D3 in satellite cells. In particular, we first observed that the D3, which is responsible for T3 inactivation, is highly expressed in satellite cells, once they have been activated, and remain active in proliferating cells. The high levels of D3 appear to be important to prevent the exposure of satellite cells to high levels of thyroid hormone, since its depletion caused T3-dependent cell death in culture, even in the proliferating satellite cells. In vivo data confirmed the toxicity of T3 excess in muscle, as demonstrated by the increase of apoptosis in satellite cells activated by cardiotoxin and contemporary exposed to T3. This observation clearly suggests that the excess of thyroid hormone might impair muscle regeneration in vivo, in fact when mice are either exposed to excess of T3 or depleted of D3, no skeletal muscle regeneration occurs after injury. These data indicate that D3 is essential for satellite cells amplification in myogenesis, representing a survival mechanism triggered by satellite cells to protect themselves from T3-induced apoptosis (• **Figure 2**) [2].

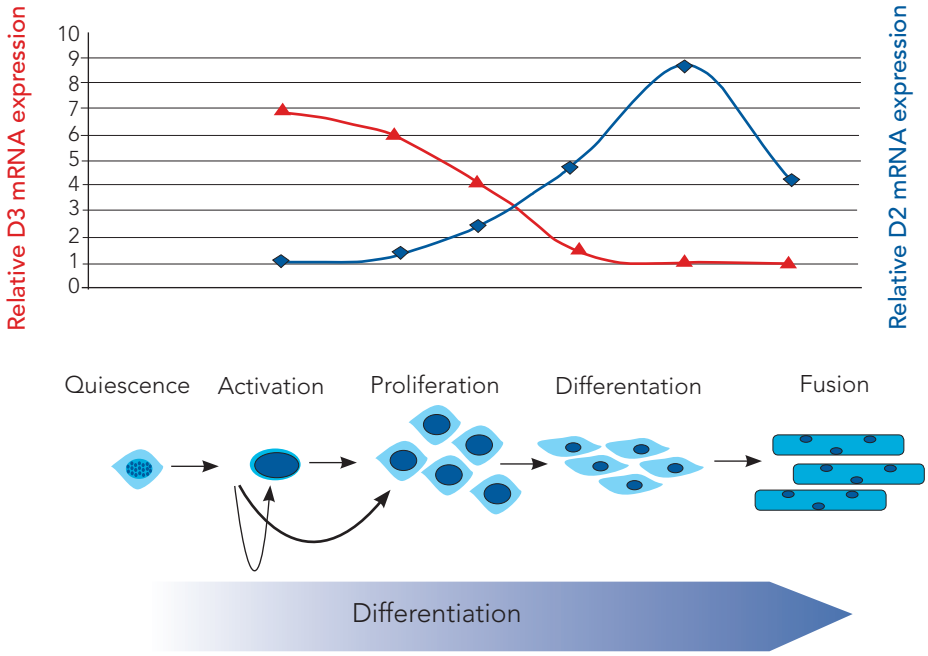
• **Figure 2.** D3 protects activated satellite cells from thyroid hormone-induced apoptosis




*Source:* Dentice *et al.*, 2014 [2].

On the other hand we observed that deiodinase 2 (D2) has an opposite expression profile in myogenesis [3]. In fact, D2 is expressed lately in myogenesis, suggesting that it could play a role in the differentiation of satellite cells. Indeed D2 knock out mice were not able to complete the muscle regeneration after damage. Moreover, no regulation of differentiative factors occurs in the mice with D2 depletion. Hence, D2 and D3 are finely and oppositely tuned during myogenesis, to strictly regulate T3 peripheral levels (• **Figure 3**).

• **Figure 3.** Deiodinase expression profile during myogenesis of satellite cells




*Source:* Dentice *et al.*, 2014 [4].

Hence, we wondered whether the intracellular control of TH could be implicated in the control of quiescence of satellite cells and if this control could represent a target to influence muscle regeneration.

To address this question we first analyzed some TH machinery key genes in the activated and quiescent cells, divided upon the different levels of Pax7 expression. Interestingly we found that D2 and D3 are differently expressed in the quiescent and activated cells, in a completely opposite fashion. Also thyroid hormone receptors and transporters are differently expressed in the two cell populations.

When D2 was depleted in culture by reverse T3 treatment, we observed a shift from Pax7 to myoD expression, suggesting a shift towards a proliferating phenotype. Similarly, when D2 was specifically depleted in satellite cells in vitro and in vivo model, we observed that proliferating cells were increased. These observations led us to the conclusion that D2 depletion is a key factor to influence the balance between quiescence and activation of satellite cells in favor of the activation. This would suggest that quiescent satellite cells have high thyroid hormone levels.

Finally, we wondered if these observations could be viable to a therapeutic strategy. We decided to study Duchenne Muscular Dystrophy. This disease affects male patients, usually in their young age and is characterized by severe muscle degeneration. A premature depletion of satellite cells results in the inability to counteract the muscle loss, which occurs in these patients due to dystrophin gene mutation. By crossing dystrophic mice with D2 ko mice, we observed that regeneration process was maintained in skeletal muscle at 3 weeks of age. This ability was unfortunately lost later, probably due to the depletion of satellite cells pool.

On the whole we can conclude that deiodinases D2 and D3 play a key role in myogenesis, being tightly and differentially regulated in myogenesis and muscle regeneration. In particular we demonstrated that D2 is expressed in quiescent satellite cells, and that its depletion can activate those cells by enhancing their proliferative potential.

Finally the loss of D2 delays the onset of muscle dystrophy, preserving satellite pool cells from exhaustion at the beginning.

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# Thyroid hormone and bone

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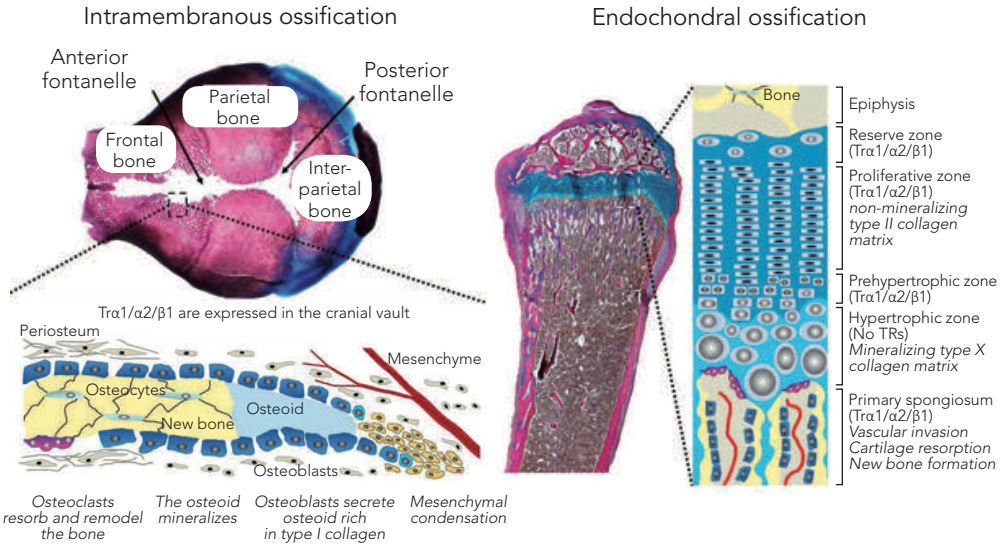
Thyroid hormones regulate skeletal development, linear growth and adult bone maintenance. In hypothyroidism endochondral ossification is delayed, growth plates are disorganised resulting in growth retardation with impaired ossification and mineralization in both the epiphyses and metaphyses. By contrast, in thyrotoxicosis there is advanced ossification and increased bone mineral deposition during growth, but short stature results from premature quiescence of the growth plates with exhaustion of stem cell reserves [1-4].

The skeleton develops by two distinct mechanisms. Intramembranous ossification is the mechanism by which the flat bones of the skull, some pelvis bones and the lateral clavicle are formed, while the long bones develop via endochondral ossification.

Intramembranous ossification begins with condensation of mesenchymal cells, which transform directly into osteoblasts and bone lining cells and secrete osteoid matrix that is rich in type 1 collagen and which mineralizes to form bone. Endochondral ossification occurs by a different process. Mesenchyme precursor cells differentiate to chondrocytes and a cartilage scaffold is formed prior to recruitment of osteoblast precursor cells and bone formation. Thyroid hormones regulate both intramembranous and endochondral ossification directly and the nuclear thyroid hormone receptors, TRα and TRβ, are expressed widely in the developing skeleton (• **Figure 1**).

In juveniles chondrocytes, which form cartilage and control the pace of linear growth, and osteoblasts, which form and mineralize bone, are both targeted directly by T3. Chondrocytes and osteoblasts express thyroid hormone transporters, deiodinase enzymes and T3 receptors, predominantly TRα. T3 acts directly in chondrocytes to regulate the pace of cell proliferation and differentiation, vascular invasion of cartilage, cartilage matrix synthesis, mineralization and degradation. Although several downstream mediators such as fibroblast growth factor receptor 3, various cell cycle

• **Figure 1.** Intramembranous and endochondral ossification

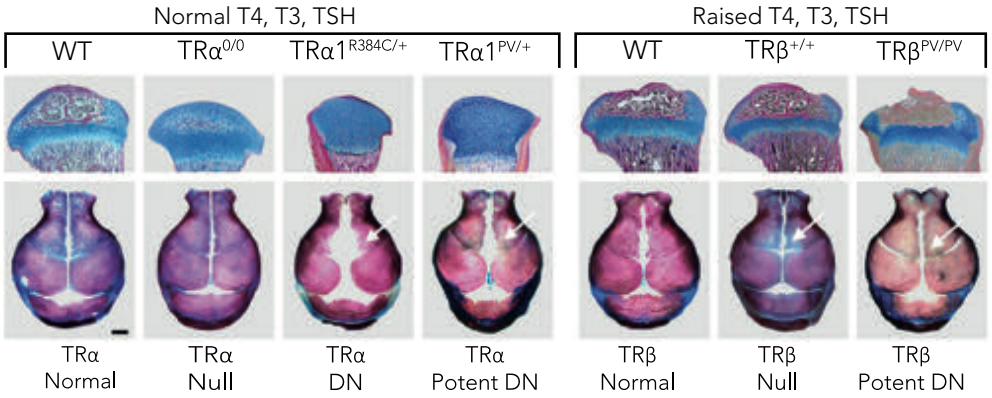



*Source:* Bassett, Williams, 2016 [5].

regulators and cytokines have been identified, the precise molecular mechanisms of T3 action in chondrocytes remain unclear. In osteoblasts T3 regulates cell proliferation and differentiation, bone matrix synthesis and mineralization, bone modeling and remodeling, and similarly the molecular mechanisms of T3 action in osteoblasts are incompletely characterised [5]. We studied mutant mice with deleted or mutant TRα or TRβ, to analyse the effects on skeletal development. In case of TRα mutation minimal effects on circulating thyroid hormone levels were observed; on the contrary mutation or deletion of TRβ leads to thyroid hormone resistance, with increases of both circulating thyroid hormone and TSH concentrations. When TRα was deleted or mutated we observed a delay in endochondral ossification, intramembranous ossification and bone age, together with a decrease in bone mineral deposition and growth retardation. On the other hand, in case of mutation or deletion of TRβ, endochondral and intramembranous ossification were accelerated, bone age was advanced and mineral deposition increased, resulting in short stature [2, 4] (• **Figure 2**). These features are characteristic of the skeletal consequences of hypothyroidism (TRα disruption) and hyperthyroidism (TRβ disruption), respectively.

Studies of humans with TR mutations are consistent with findings in mutant mice. Individuals with TRα mutations display skeletal dysplasia due to delayed endochondral and intramembranous ossification, which is characterised by short stature, macrocephaly, patent skull sutures, delayed tooth eruption and epiphyseal dysgenesis. These skeletal abnormalities are similar to those observed in congenital hypothyroidism [6, 7].

• **Figure 2.** Consequences of TR mutations in juvenile mice

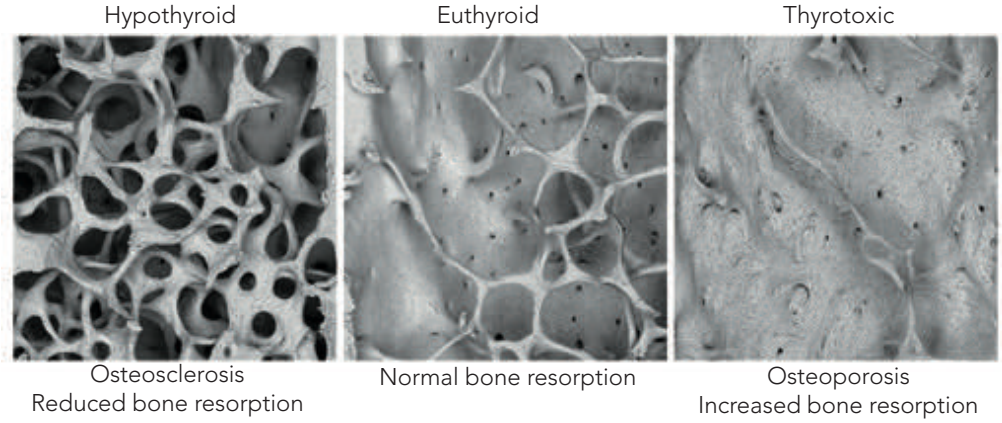



*Source:* Bassett, Williams, 2016 [5].

Although the skeletal consequences of TRβ mutation in humans have not been studied in detail, features of delayed or accelerated development have been described and this is likely explained by the specific properties of the individual mutations studied and confounded by genetic background factors and heterogeneous methods to assess the skeleton. Overall, mutations of TRα result in impaired T3 action in skeletal cells and result in a phenotype that is similar to hypothyroidism, whereas the consequences of TRβ mutation result from disruption of the hypothalamic-pituitary-thyroid axis leading to elevated thyroid hormone levels and a phenotype similar to the consequences of thyrotoxicosis [5].

Thyroid hormones also have important actions in the adult skeleton. Thyrotoxicosis and subclinical hyperthyroidism are associated with accelerated bone loss and an increased susceptibility to fragility fracture in prospective studies. Maintenance of bone mass, mineralisation and strength are regulated by the bone remodeling cycle. During this process, osteocytes buried within bone tissue function as mechanical sensors that respond to mechanical strain and micro-damage by releasing growth factors or undergoing apoptosis to attract monocytes and initiate osteoclastogenesis. Mature osteoclasts resorb damaged bone and release chemo-attractants from the dissolving bone matrix to stimulate migration of osteoblasts to the resorbed surface. Osteoblasts then deposit new osteoid and mineralise the matrix, ultimately replacing the resorbed damaged bone with new bone. Homeostatic control of this continuous process of repair and renewal of bone is regulated by thyroid hormones. However, in hypothyroidism reduced bone turnover leads to a decrease in bone resorption and gradual accumulation of bone that is demonstrated by osteosclerosis in animal models, whereas in hyperthyroidism increased osteoclastic bone resorption results in accelerated bone loss and osteoporosis (• **Figure 3**).

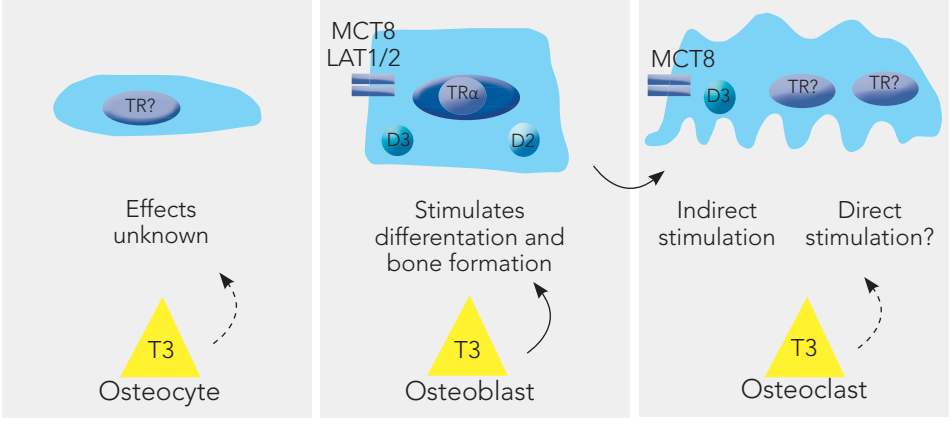
• **Figure 3.** Thyroid hormones increase osteoclastic bone resorption




*Source:* Bassett, Williams, 2016 [5].

Despite this understanding, it is still unclear which cells are direct T3 targets in the adult skeleton. It remains unknown whether T3 exerts direct actions in osteocytes. Although it has been established that T3 acts directly via TRα in osteoblasts to stimulate cell differentiation, bone formation and bone mineralization the molecular mechanisms underlying these process have not yet been defined. It also remains unclear whether T3 acts directly in osteoclasts to stimulate bone resorption, or whether these effects are indirect and mediated via primary T3 actions in another cell lineage (• **Figure 4**).

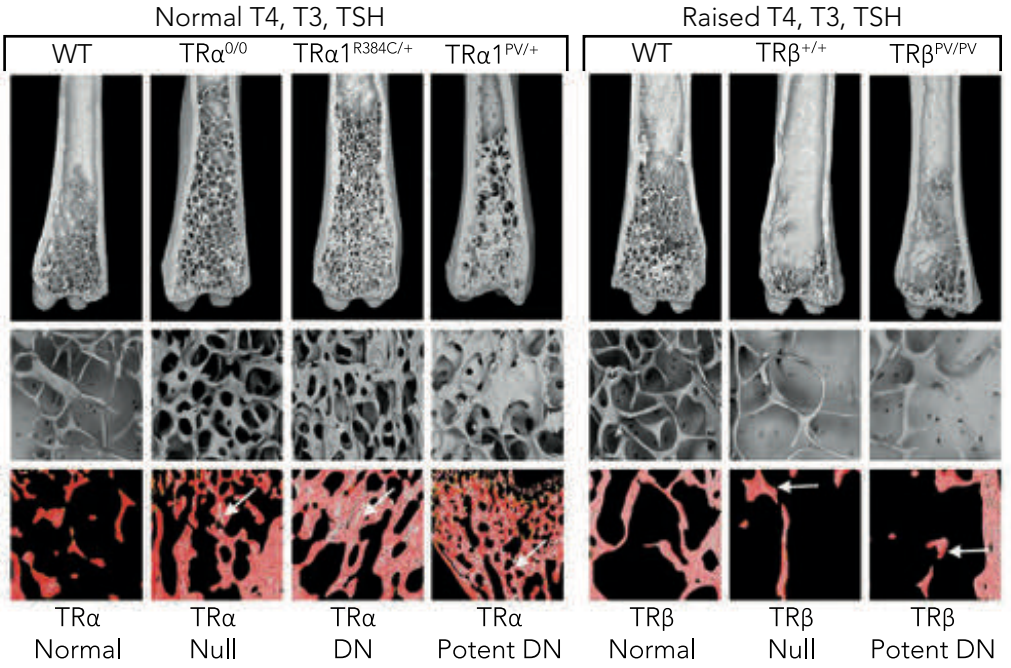
• **Figure 4.** T3 target cells in adult bone




*Source:* Bassett, Williams, 2016 [5].

In TR mutant mice adults with TRα and TRβ mutations display opposite phenotypes. Thus, TRα mutation results in increased bone mass and mineralization due to impaired thyroid hormone action in bone. By contrast TRβ mutants display elevated TH concentrations due to disruption of the hypothalamic-pituitary axis that result in increased bone turnover, increased osteoclastic bone resorption and osteoporosis [3, 4, 8] (• **Figure 5**). In humans with TR mutations, phenotypes are also consistent with findings in mutant mice. Again very few patients with TRβ mutations have been investigated thoroughly, and skeletal phenotypes are variable. On the other hand in the case of TRα mutations, persistent skeletal dysplasia and short stature is observed [6, 7, 9-12].

• **Figure 5.** Consequences of TR mutations in adult mice




*Source:* Bassett, Williams, 2016 [5].

Finally, in population studies it has been shown that even when thyroid function is in the upper part of the normal reference range, there is an association with reduced bone mineral density and an increased risk of incident fracture in healthy euthyroid postmenopausal women [13]. Recently, an individual participant meta-analysis of prospective studies (13 prospective cohorts, 70,298 patients, 762,401 person-years of follow-up)

revealed that subclinical hyperthyroidism results in an increased relative risk of fracture [14]. It is thus essential for both skeletal development and adult bone mass and strength that adequate and normal euthyroid hormone levels are maintained throughout life.

Overall, TRα 1 is the principal mediator of T3 action in the skeleton in both humans and mice. The actions of T3 are anabolic during postnatal growth, but catabolic in the adult skeleton. T3 acts directly in chondrocytes and osteoblasts, whereas the potential actions of T3 in osteocytes and osteoclasts require further study and are not yet understood.

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# Conclusions

Thyroid hormones are well known to target many tissues and organs, their action being mediated by several TH machinery components, such as thyroid hormone transporters, thyroid hormone receptors, and the deiodinases, specific enzymes involved in their metabolism. Every factor belonging to this machinery can potentially be determinant in the specific tissue response to thyroid hormone.

Despite the circulating levels of thyroid hormones, it is then very important to consider the local levels of thyroid hormones in defining specific tissue homeostasis or pathologies. From this point of view deiodinases, able to define the local levels of thyroid hormones, are key players in peripheral regulation by thyroid.

In fact, deiodinase 2 ability to define T3 local levels was demonstrated to be crucial in liver, since the disruption of D2-mediated T3 signaling protects adult mice from diet-induced obesity, steatosis and hypertriglyceridemia. Dio2 again is important in the reproduction regulation by season in mammals, birds and fishes, where, together with TSH, can trigger some specific signaling transduction conserved throughout different species. On the other hand, Dio3 play a crucial role in skeletal muscle regeneration. Its ability to control T3 local levels protects satellite cells from thyroid hormone excess induced apoptosis, thus driving the regeneration capability of skeletal muscle. Dio3 is also involved in heart failure, where its overexpression prevents the T3 ability to break pathological remodeling after injury.

Even the thyroid hormone receptors play key roles in the peripheral action of thyroid hormones, being relevant in the bones, where TRα1 mediates T3 action on skeleton development and remodeling. TRα1 is also crucial in mediating T3 action in brain development and function, as well as in neural differentiation; whereas the β1 isoform is relevant in retinal function.

We can then conclude that to clarify the specific local action of THs represents today an intriguing futurable therapeutic approach, to ameliorate various organ diseases.

Purpose of the Forum "The thyroid… in the periphery!", organized in Naples by IBSA Foundation with the collaboration of Domenico Salvatore, Federico II University, was to discuss about thyroid not usually looking at its diseases, but from a new point of view, inside the function of thyroid hormones and with a systematic vision, involving diseases affecting other organs than thyroid.

World leading endocrinology experts deepened the specific roles of thyroid hormones in different tissues focusing on the new perspective of considering the local action of thyroid hormones as a possible therapeutic approach for various diseases.